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1946/1A483-US6

ACTIVE AGENT TRANSPORT SYSTEMS

10

This application is a continuation-in-part of:

(a) U.S. Serial No. 08/763,183, filed December 10, 1996, which is a continuation-in-part of U.S. Serial No. 08/328,932, filed October 25, 1994;

(b) U.S. Serial No. 08/051,019, filed April 22, 1993, now U.S. Patent  
 15 5,451,410;

(c) U.S. Serial No. 08/168,776, filed December 16, 1993, now U.S. Patent 5,447,728, which is a continuation-in-part of U.S. Serial No. 08/051,019, filed April 22, 1993, now U.S. Patent 5,451,410, and of U.S. Serial No. 08/143,571, filed October 26, 1993, which is a continuation-in-part  
 20 of U.S. Serial No. 08/076,803, filed June 14, 1993, which is a continuation-in-part of U.S. Serial No. 07/920,346, filed July 27, 1992, now U.S. Patent 5,443,841, which is a continuation-in-part of U.S. Serial No. 07/898,909, filed June 15, 1992;

(d) PCT Serial No. PCT/US94/04560, filed April 22, 1994, which is a  
 25 continuation-in-part of U.S. Serial No. 08/051,019, filed April 22, 1993, now U.S. Patent 5,451,410, and of U.S. Serial No. 08/205,511, filed on March 2, 1994;

(e) U.S. Serial No. 08/231,622, filed April 22, 1994;

(f) U.S. Serial No. 08/205,511, filed March 2, 1994;

(g) U.S. Serial No. 08/231,623, filed April 22, 1994, now U.S. Patent  
 30 5,541,155;

- (h) U.S. Serial No. 08/315,200, filed September 29, 1994;  
(i) U.S. Serial No. 08/316,404, filed September 30, 1994; and  
(j) U.S. Serial No. 08/820,644, filed March 18, 1997, which is a  
conversion of U.S. provisional patent application Serial No. 60/017,902, filed  
5 March 29, 1996.

### **FIELD OF THE INVENTION**

The present invention relates to methods and compositions for  
transporting active agents, and particularly biologically active agents, across cell  
10 membranes or lipid bilayers. These methods and compositions facilitate the  
delivery of an active agent to a target, such as the delivery of a pharmaceutical  
agent through an adverse environment to a particular location of the body.

### **BACKGROUND OF THE INVENTION**

15 Conventional means for delivering active agents to their intended  
targets, e.g. human organs, tumor sites, etc., are often severely limited by the  
presence of biological, chemical, and physical barriers. Typically, these barriers  
are imposed by the environment through which delivery must take place, the  
environment of the target for delivery, or the target itself.

20 Biologically active agents are particularly vulnerable to such  
barriers. In the delivery to animals of such agents, including, but not limited to  
pharmacological and therapeutic agents, barriers are impeded by the body.  
Subcutaneous, nasal or sublingual delivery to the circulatory system for many  
biologically active agents would be the route of choice for administration to  
25 animals if not for physical barriers such as the skin, lipid bi-layers, and various  
organ membranes that are relatively impermeable to certain biologically active  
agents, but one or more of which must be traversed before an agent delivered  
via these routes can reach the circulatory system. Additionally, delivery such  
as, for example, sublingual delivery may be impeded by chemical barriers such  
30 as the varying pH in the gastrointestinal (GI) tract and the presence of powerful  
digestive enzymes.

While in many cases different methods of administration of these compounds would be preferable. Many of these agents cannot be delivered by these routes to the target at which the active agent renders its intended biological effect.

Typically, the initial focus of drug design is on the physiochemical properties of pharmaceutical compounds and particularly their therapeutic function. The secondary design focus is on the need to deliver the active agent to its biological target(s). This is particularly true for drugs and other biologically active agents that are designed for oral administration to humans and other animals. However, thousands of therapeutic compounds are discarded because no delivery systems are available to ensure that therapeutic titers of the compounds will reach the appropriate anatomical location or compartment(s) after administration and particularly oral administration. Furthermore, many existing therapeutic agents are underutilized for their approved indications because of constraints on their mode(s) of administration. Additionally, many therapeutic agents could be effective for additional clinical indications beyond those for which they are already employed if there existed a practical methodology to deliver them in appropriate quantities to the appropriate biological targets.

Although nature has achieved successful inter- and intra-cellular transport of active agents such as proteins, this success has not been translated to drug design. In nature, the transportable conformation of an active agent such as a protein is different than the conformation of the protein in its native state. In addition, natural transport systems often effect a return to the native state of the protein subsequent to transport. When proteins are synthesized by ribosomes, they are shuttled to the appropriate cellular organelle by a variety of mechanisms e.g. signal peptides and/or chaperonins. Gething, M-J., Sambrook, J., *Nature*, 355, 1992, 33-45. One of the many functions of either the signal peptides or the chaperonins is to prevent premature folding of the protein into

the native state. The native state is usually described as the 3-dimensional state with the lowest free energy. By maintaining the protein in a partially unfolded state, the signal peptides or the chaperonins facilitate the protein's ability to cross various cellular membranes until the protein reaches the appropriate  
 5 organelle. The chaperonin then separates from the protein or the signal peptide is cleaved from the protein, allowing the protein to fold to the native state. It is well known that the ability of the protein to transit cellular membranes is at least partly a consequence of being in a partially unfolded state.

Current concepts of protein folding suggest that there are a number  
 10 of discrete conformations in the transition from the native state to the fully denatured state. Baker, D., Agard, D.A., *Biochemistry*, 33, 1994, 7505-7509. The framework model of protein folding suggests that in the initial early stages of folding the domains of the protein that are the secondary structure units will form followed by the final folding into the native state. Kim, P.S., Baldwin, R.L.,  
 15 *Annu. Rev. Biochem.*, 59, 1990, 631-660. In addition to these kinetic intermediates, equilibrium intermediates appear to be significant for a number of cellular functions. Bychkova, V.E., Berni, R., et al, *Biochemistry*, 31, 1992, 7566-7571, and Sinev, M.A., Razgulyaev, O.I., et al, *Eur. J. Biochem.*, 1989, 180, 61-66. Available data on chaperonins indicate that they function, in part,  
 20 by keeping proteins in a conformation that is not the native state. In addition, it has been demonstrated that proteins in partially unfolded states are able to pass through membranes, whereas the native state, especially of large globular proteins, penetrates membranes poorly, if at all. Haynie, D.T., Freire, E., *Proteins: Structure, Function and Genetics*, 16, 1993, 115-140.

25 Similarly, some ligands such as insulin which are unable to undergo conformational changes associated with the equilibrium intermediates described above, lose their functionality. Hua, Q. X., Ladbury, J.E., Weiss, M.A., *Biochemistry*, 1993, 32, 1433-1442; Remington, S., Wiegand, G., Huber, R.,

1982, 158, 111-152; Hua, Q. X., Shoelson, S.E., Kochoyan, M. Weiss, M.A.,  
*Nature*, 1991, 354, 238-241.

Studies with diphtheria toxin and cholera toxin indicate that after  
 diphtheria toxin binds to its cellular receptor, it is endocytosed, and while in this  
 5 endocytic vesicle, it is exposed to an acidic pH environment. The acidic pH  
 induces a structural change in the toxin molecule which provides the driving  
 force for membrane insertion and translocation to the cytosol. See, Ramsay, G.,  
 Freire, E. *Biochemistry*, 1990, 29, 8677-8683 and Schon, A., Freire, E.,  
*Biochemistry*, 1989, 28, 5019 - 5024. Similarly, cholera toxin undergoes a  
 10 conformational change subsequent to endocytosis which allows the molecule to  
 penetrate the nuclear membrane. See also, Morin, P.E., Diggs, D., Freire, E.,  
*Biochemistry*, 1990, 29, 781-788.

Earlier designed delivery systems have used either an indirect or a  
 direct approach to delivery. The indirect approach seeks to protect the drug from  
 15 a hostile environment. Examples are enteric coatings, liposomes, microspheres,  
 microcapsules. See, colloidal drug delivery systems, 1994, ed. Jorg Freuter,  
 Marcel Dekker, Inc.; U.S. Patent No. 4,239,754; Patel et al. (1976), *FEBS*  
*Letters*, Vol. 62, pg. 60; and Hashimoto et al. (1979), *Endocrinology Japan*, Vol.  
 26, pg. 337. All of these approaches are indirect in that their design rationale is  
 20 not directed to the drug, but rather is directed to protecting against the  
 environment through which the drug must pass enroute to the target at which it  
 will exert its biological activity, i.e. to prevent the hostile environment from  
 contacting and destroying the drug.

The direct approach is based upon forming covalent linkages with  
 25 the drug and a modifier, such as the creation of a prodrug. Balant, L.P., Doelker,  
 E., Buri, P., *Eur. J. Drug Metab. And Pharmacokinetics*, 1990, 15(2), 143-153.  
 The linkage is usually designed to be broken under defined circumstances, e.g.  
 pH changes or exposure to specific enzymes. The covalent linkage of the drug  
 to a modifier essentially creates a new molecule with new properties such as an

altered log P value and/or as well as a new spatial configuration. The new molecule has different solubility properties and is less susceptible to enzymatic digestion. An example of this type of method is the covalent linkage of polyethylene glycol to proteins. Abuchowski, A., Van Es, T., Palczuk, N.C.,

5 Davis, F.F., *J. Biol. Chem.* 1977, 252, 3578.

Broad spectrum use of prior delivery systems has been precluded, however, because: (1) the systems require toxic amounts of adjuvants or inhibitors; (2) suitable low molecular weight cargos, i.e. active agents, are not available; (3) the systems exhibit poor stability and inadequate shelf life; (4) the  
10 systems are difficult to manufacture; (5) the systems fail to protect the active agent (cargo); (6) the systems adversely alter the active agent; or (7) the systems fail to allow or promote absorption of the active agent.

There is still a need in the art for simple, inexpensive delivery systems which are easily prepared and which can deliver a broad range of active  
15 agents to their intended targets, especially in the case of pharmaceutical agents that are to be administered via the oral route.

### SUMMARY OF THE INVENTION

The present invention discloses methods for administering, by either  
20 the subcutaneous, nasal, or sublingual routes, a biologically active agent to a subject in need of such agent. A first method includes the steps of:

(a) providing a biologically active agent which can exist in a native conformational state, a denatured conformational state, and an intermediate conformational state which is reversible to the native state and which is  
25 conformationally between the native and denatured states;

(b) exposing the biologically active agent to a complexing perturbant to reversibly transform the biologically active agent to the intermediate state and to form a transportable supramolecular complex wherein the perturbant is in an

amount effective for subcutaneous, nasal or sublingual delivery of the biologically active agent; and

(c) either subcutaneously, nasally, or sublingually administering the supramolecular complex to the subject.

- 5           The perturbant has a molecular weight between about 150 and about 600 daltons, and contains at least one hydrophilic moiety and at least one hydrophobic moiety. The supramolecular complex comprises the perturbant non-covalently bound or complexed with the biologically active agent. In the present invention, the biologically active agent does not form a microsphere after  
10 interacting with the perturbant.

Also contemplated is a method for preparing subcutaneously, nasally, or sublingually administrable biologically active agent comprising steps (a) and (b) above.

- 15           In alternate embodiments, subcutaneous, nasal, or sublingual delivery compositions are provided. The compositions comprise a supramolecular complex including:

- (a) a biologically active agent in an intermediate conformational state which is reversible to the native state, non-covalently complexed with  
(b) a complexing perturbant having a molecular weight ranging  
20 from about 150 to about 600 and having at least one hydrophilic moiety and at least one hydrophobic moiety wherein the perturbant is in an amount effective for subcutaneous, nasal, or sublingual delivery of the biologically active agent;  
and

- wherein the intermediate state is conformationally between  
25 the native conformation state and denatured conformation state of the biologically active agent and the composition is not a microsphere.

Further contemplated is a method for preparing an agent which is a mimetic which is capable of being administered, by the subcutaneous, nasal, or sublingual route, to a subject in need of such agent. A biologically active agent

which can exist in a native conformational state, a denatured conformational state, and an intermediate conformational state which is reversible to the native state and which is conformationally between the native state and the denatured state, is exposed to a complexing perturbant in an amount effective for

5 subcutaneous, nasal, or sublingual delivery of such an agent to reversibly transform the biologically active agent to the intermediate conformational state and to form a transportable supramolecular complex. The perturbant has a molecular weight between about 150 and about 600 daltons and at least one hydrophilic moiety and one hydrophobic moiety. The supramolecular complex  
10 comprises the perturbant non-covalently complexed with the biologically active agent, and the biologically active agent does not form a microsphere with the perturbant. A mimetic of the supramolecular complex is prepared.

Alternatively, a method for preparing an agent which is capable of being administered either by the subcutaneous, nasal, or sublingual routes, is  
15 provided. A biologically active agent which can exist in a native conformational state, a denatured conformational state, and an intermediate conformational state which is reversible to the native state and which is conformationally between the native and denatured states, is exposed to a perturbant to reversibly transform the biologically active agent to the intermediate state. The perturbant  
20 is in a respective amount effective for subcutaneous, nasal, or sublingual delivery of the agent. The agent, a mimetic of the intermediate state, is prepared.

The administration by these routes in the methods and compositions of the present invention results in delivery of the agent in bioavailable and bioactive form.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is illustration of a native gradient gel of  $\alpha$ -interferon (IFN) and a modified amino acid complexing perturbant.



Figure 2 is an illustration of a native gradient gel of  $\alpha$ -interferon and a thermal condensate complexing perturbant.

Figure 3 is a graphic illustration of guanidine hydrochloride (GuHCl) induced denaturation of  $\alpha$ -interferon.

5                    Figure 4 is a graphic illustration of the concentration effect of GuHCl on  $\alpha$ -interferon conformation.

Figure 5 is a graphic illustration of the pH denaturation of  $\alpha$ -interferon.

Figure 6 is a graphic illustration of the pH denaturation of insulin.

10                   Figures 7A and 7B are graphic illustrations of the reversibility of the circular dichroism spectrum of  $\alpha$ -interferon.

Figure 8 is a graphic illustration of the circular dichroism spectrum of  $\alpha$ -interferon.

15                   Figure 9 is a graphic illustration of intrinsic tryptophan fluorescence of  $\alpha$ -interferon and a complexing perturbant.

Figure 10 is a graphic illustration of the differential scanning calorimetry of  $\alpha$ -interferon and complexing perturbant.

Figures 11A and 11B are graphic illustrations of the reversibility of the transformation due to complexing perturbants.

20                   Figure 12 is a graphic illustration of the effect of complexing perturbant on  $\alpha$ -interferon.

Figure 13 is a graphic illustration of the concentration effect of complexing perturbant on  $\alpha$ -interferon conformation.

25                   Figure 14 is a graphic illustration of the effect of complexing perturbant on  $\alpha$ -interferon.

Figure 15 is a graphic illustration of the Isothermal Titration Calorimetry of  $\alpha$ -interferon and complexing perturbant.

Figure 16 is a graphic illustration of the Isothermal Titration Calorimetry of  $\alpha$ -interferon and complexing perturbant.

Figure 17 is a graphic illustration of the effects of complexing perturbants on  $\alpha$ -interferon.

Figure 18 is a graphic illustration of the effect of the concentration of complexing perturbants on  $\alpha$ -interferon.

5            Figure 19 is a graphic illustration of the Isothermal Titration Calorimetry of  $\alpha$ -interferon and complexing perturbant.

Figure 20 is a graphic illustration of pancreatic inhibition assay with  $\alpha$ -interferon and complexing perturbants.

10          Figure 21 is a graphic illustration of the effect of DSC of heparin at pH 5.0.

Figure 22 is a graphic illustration of the DSC of DPPC with perturbant compounds at several concentrations (units  $T_M$ , °C vs. concentration).

Figure 23 is a graphic illustration of the concentration effect of complexing perturbant compound L on the DPPC conformation.

15          Figure 24 is a graphic illustration of the concentration effect of complexing perturbant compound L on rhGH conformation.

Figure 25 is a graphic illustration of the concentration effect of complexing perturbant compound LI on rhGH conformation.

20          Figure 26 is a graphic illustration of the concentration effect of complexing perturbant compound XI on rhGH conformation.

Figure 27 is a graphic illustration of the differential light scattering of perturbant compound L in a 10mM phosphate buffer at pH 7.0.

Figure 28 is a graphic illustration of the results of subcutaneous injection of rhGH composition in rats.

25          Figure 29 is a graphic illustration of the results of Sublingual (SL), intranasal (IN), and intracolonic (IC) dosing of rhGH in rats.

Figure 30 is a graphic illustration of the results of intracolonic dosing of heparin with compound XXXI carrier.

## DETAILED DESCRIPTION OF THE INVENTION

Subcutaneous, sublingual, and intranasal coadministration of an active agent, such as recombinant human growth hormone (rhGH), and the delivery agents, and particularly proteins, described herein results in an increased bioavailability of the active agent compared to administration of the active agent alone. A similar result is obtained by coadministration of salmon calcitonin with the delivery agents, in rats. Data supporting these findings are presented in the examples.

The present methods effect active agent delivery by creating a reversibly non-covalently complexed supramolecule from the active agent and an amount of complexing perturbant appropriate for the route of delivery. As a result, the three-dimensional structure or conformation of the active agent is changed, but the chemical composition of the active agent molecule is not altered. This alteration in structure (but not composition) provides the active agent with the appropriate properties, such as, for example, solubility (log P) to cross or penetrate a physical or chemical barrier, membrane, or lipid bilayer, to resist enzymatic degradation and the like. Crossing refers to transport from one side of the cell membrane or lipid bilayer to the opposite side (i.e. from the outside or exterior to the inside or interior of a cell and/or visa versa), whether the cell membrane or lipid bilayer is actually penetrated or not. Additionally, the perturbed intermediate state of the active agent or the supramolecular complex itself can be used as a template for the preparation of mimetics which, accordingly, could be delivered to a target by the appropriate route, i.e., subcutaneous, nasal, or sublingual. After crossing the cell membrane or lipid bilayer, an active agent has biological activity and bioavailability, either by restoration to the native state or by retaining biological activity or bioavailability acquired in the intermediate state. The mimetic acts similarly after crossing the cell membrane or lipid bilayer.

### Active Agents

The native conformational state of an active agent is typically described as the three dimensional state with the lowest free energy ( $\Delta G$ ). It is the state in which the active agent typically possesses the full complement of activity ascribed to the agent, such as the full complement of biological activity ascribed to a biologically active agent.

The denatured conformational state is the state in which the active agent has no secondary or tertiary structure.

Intermediate conformational states exist between the native and denatured states. A particular active agent may have one or more intermediate states. The intermediate state achieved by the present invention is structurally and energetically distinct from both the native and denatured states. Active agents useful in the present invention must be transformable from their native conformational state to an intermediate conformational state which can be administered by the route of choice and back to their native state, i.e. reversibly transformable, so that when the active agent reaches its target, such as when a delivered drug reaches the circulatory system, the active agent retains, regains, or acquires a biologically, pharmacologically, or therapeutically significant complement of its desired biological activity. Preferably the  $\Delta G$  of the intermediate state ranges from about -20 Kcal/mole to about 20 Kcal/mole, and most preferably, it ranges from about -10 Kcal/mole to about 10 Kcal/mole, all relative to the native state.

For example in the case of a protein, the intermediate state has significant secondary structure, significant compactness due to the presence of a sizable hydrophobic core, and a tertiary structure reminiscent of the native fold but without necessarily exhibiting the packing of the native state. The difference in free energy ( $\Delta G$ ) between the intermediate state and the native state is relatively small. Hence, the equilibrium constant between the native and the transportable, reversible intermediate state(s) is close to unity (depending upon

experimental conditions). Intermediate states can be confirmed by, for example, differential scanning calorimetry (DSC), isothermal titration calorimetry (ITC), native gradient gels, NMR, fluorescence, and the like.

Without being bound by any theory, applicants believe that the physical chemistry of the intermediate state can be understood by the following explanation relating to proteinaceous active agents. Proteins can exist in stable intermediate conformations that are structurally and energetically distinct from either the native state or the denatured state. The inherent stability of any conformation(s) of any protein is reflected in the Gibbs free energy of the conformation(s). The Gibbs free energy for any state of a monomeric protein is described thermodynamically by the following relationship:

$$\Delta G^0 = \Delta H^0(T_R) - T\Delta S^0(T_R) + \Delta C_p^0 ( (T-T_R) - T \ln(T/T_R) ) \quad (1)$$

where  $T$  is the temperature,  $T_R$  is a reference temperature,  $\Delta H^0(T_R)$  and  $T\Delta S^0(T_R)$  are the relative enthalpy and entropy of this state at the reference temperature, and  $\Delta C_p^0$  is the relative heat capacity of this state. It is convenient to choose the native state as the reference state to express all relative thermodynamic parameters.

The sum of the statistical weights of all states accessible to the protein is defined as the partition function  $Q$ :

$$Q = \sum_{i=0}^n e^{-\Delta G_i^0/RT} \quad (2)$$

Equation 2 can also be written as

$$Q = 1 + \sum_{i=1}^{n-1} e^{-DG_i/RT} + e^{-DG_n/RT} \quad (3)$$

where the second term includes all the intermediates that become populated during the transition. The first and last terms of equation (3) are the statistical weights of the native and denatured states, respectively. Under most conditions, protein structure could be approximated by a two-state transition function:

$$Q \approx 1 + e^{-\Delta G_n/RT} \quad (4)$$

See, Tanford, C., *Advances in Protein Chemistry*, 1968, 23, 2-95. Conformations of proteins that are intermediate between the native state and the denatured state can be detected by, for example, NMR, calorimetry, and fluorescence. Dill, K.A., Shortle, D., *Annu. Rev. Biochem.* 60, 1991, 795-825.

All thermodynamic parameters can be expressed in terms of the partition function. Specifically the population of molecules in state *i* is given in equation (5)

$$P_i = \frac{e^{-\Delta G_i/RT}}{Q} \quad (5)$$

Therefore, measurement of the appropriate terms in equation (1) that would allow for the calculation of the Gibbs free energy would determine the extent to which any intermediate state(s) is populated to any significant degree under defined experimental conditions. This in turn indicates the role that these intermediate state(s) play in drug delivery. The more populated the intermediate state, the more efficient the delivery.

Active agents suitable for use in the present invention include biologically active agents and chemically active agents, including, but not limited to, fragrances, as well as other active agents such as, for example, cosmetics.

Biologically active agents include, but are not limited to, pesticides, pharmacological agents, and therapeutic agents. For example, biologically active agents suitable for use in the present invention include, but are not limited to, peptides, and particularly small peptides; hormones, and particularly hormones which by themselves do not or only pass slowly through the gastro-intestinal mucosa and/or are susceptible to chemical cleavage by acids and enzymes in the gastro-intestinal tract; polysaccharides, and particularly mixtures of muco-polysaccharides; carbohydrates; lipids; or any combination thereof. Further examples include, but are not limited to, human growth hormones; bovine growth hormones; growth releasing hormones; interferons; interleukin-1; insulin; heparin, and particularly low molecular weight heparin; calcitonin; erythropoietin; atrial natriuretic factor; antigens; monoclonal antibodies; somatostatin; adrenocorticotropin, gonadotropin releasing hormone; oxytocin; vasopressin; cromolyn sodium (sodium or disodium chromoglycate); vancomycin; desferrioxamine (DFO); anti-microbials, including, but not limited to anti-fungal agents; or any combination thereof.

The methods and compositions of the present invention may combine one or more active agents.

### **Perturbants**

Perturbants serve two purposes in the present invention. In a first embodiment, the active agent is contacted with an amount of perturbant which reversibly transforms the active agent from the native state to the intermediate state suitable for administration. The perturbant, in the appropriate amount, non-covalently complexes with the active agent to form a supramolecular complex which can be administered by a selected route. This supramolecular complex

can be used as a template for the design of a mimetic or can be used as a delivery composition itself. The perturbant, in effect, fixes the active agent in the transportable intermediate state. The perturbant can be released from the supramolecular complex, such as by dilution in the circulatory system, so that

5 the active agent can return to the native state appropriate for the route of delivery. Preferably, these perturbants have at least one hydrophilic (i.e. readily soluble in water, such as for example, a carboxylate group) and at least one hydrophobic moiety (i.e. readily soluble in an organic solvent such as, for example, a benzene group), and have a molecular weight ranging from about 150

10 to about 600 daltons and most preferably from about 200 to about 500 daltons.

Complexing perturbant compounds include, but are not limited to proteinoids including linear, non-linear, and cyclic proteinoids; modified (acylated or sulfonated) amino acids, poly amino acids, and peptides; modified amino acid, poly amino acid, or peptide derivatives (ketones or aldehydes);

15 diketopiperazine/amino acid constructs; carboxylic acids; and various other perturbants discussed below.

Again without being bound by any theory, applicant believes that the non-covalent complexing may be effected by intermolecular forces including but not limited to, hydrogen bonding, hydrophilic interactions, electrostatic

20 interactions, and Van der Waals interactions. For any given active agent/perturbant supramolecular complex, there will exist some combination of the aforementioned forces that maintain the association.

The association constant  $K_a$  between the perturbant and the active agent can be defined according to equation (6)

25

$$K_a = e^{-\Delta G/RT} \quad (6)$$



The dissociation constant  $K_d$  is the reciprocal of  $K_a$ . Thus measurement of the association constants between perturbant and active agent at a defined temperature will yield data on the molar Gibbs free energy which allows for the determination of the associated enthalpic and entropic effects.

- 5 Experimentally these measurements can be made, for example, using NMR, fluorescence or calorimetry.

This hypothesis can be illustrated with proteins in the following manner:

- 10 Protein unfolding can be described according to the equilibrium that exists between its various conformational states, e.g.



- 15 where N is the native state, I is the intermediate state(s), D is the denatured state, and  $k_1$  and  $k_2$  are the respective rate constants.  $K_1$  and  $K_2$  are the respective equilibrium constants. Accordingly,

$$Q = \sum_{i=0}^n e^{-\Delta G_i/RT} \quad (2)$$

20

$$= 1 + e^{-\Delta G_1/RT} + e^{-\Delta G_2/RT} \\ = 1 + K_1 + K_2 \quad (8)$$

25

$$= 1 + k_1 + k_1 k_2 \quad (9)$$

This suggests that increasing the partition function of the intermediate state(s) should have a positive impact on the ability to deliver the active agent, i.e.

$$P_I = \frac{K_1}{(1 + K_1 + K_2)} \quad (10)$$

5

Because complexing must be reversible, the complexing of the perturbant with the active agent, as measured by the  $K_a$ , must be strong enough to insure delivery of the drug either to the systemic circulation and/or to the target(s), but not so strong so that disengagement of the perturbant will not occur in a timely manner to allow the active agent to renature if necessary to produce the desired effect(s).

10

In a second embodiment, amounts of perturbants appropriate for the route of delivery reversibly transform the active agent to the intermediate state so that the conformation of that state can be used as a template for the preparation of mimetics. Perturbants for this purpose need not, but may, complex with the active agent. Therefore, in addition to the complexing perturbants discussed above, perturbants that change the pH of the active agent or its environment, such as for example, strong acids or strong bases; detergents; perturbants that change the ionic strength of the active agent or its environment; other agents such as for example, guanidine hydrochloride; and temperature can be used to transform the active agent. Either the supramolecular complex or the reversible intermediate state can be used as a template for mimetic design.

15

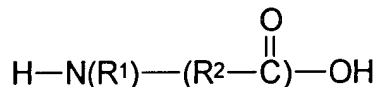
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25

### Complexing Perturbants

Amino acids are the basic materials used to prepare many of the complexing perturbants useful in the present invention. An amino acid is any carboxylic acid having at least one free amine group and includes naturally occurring and synthetic amino acids. The preferred amino acids for use in the present invention are  $\alpha$ -amino acids, and most preferably are naturally occurring  $\alpha$ -amino acids. Many amino acids and amino acid esters are readily available from a number of commercial sources such as Aldrich Chemical Co. (Milwaukee, WI, USA); Sigma Chemical Co. (St. Louis, MO, USA); and Fluka Chemical Corp. (Ronkonkoma, NY, USA).

Representative, but not limiting, amino acids suitable for use in the present invention are generally of the formula



wherein:  $\text{R}^1$  is hydrogen,  $\text{C}_1\text{-C}_4$  alkyl, or  $\text{C}_2\text{-C}_4$  alkenyl;

$\text{R}^2$  is  $\text{C}_1\text{-C}_{24}$  alkyl,  $\text{C}_2\text{-C}_{24}$  alkenyl,  $\text{C}_3\text{-C}_{10}$  cycloalkyl,  $\text{C}_3\text{-C}_{10}$  cycloalkenyl, phenyl, naphthyl,  $(\text{C}_1\text{-C}_{10}$  alkyl) phenyl,  $(\text{C}_2\text{-C}_{10}$  alkenyl) phenyl,  $(\text{C}_1\text{-C}_{10}$  alkyl) naphthyl,  $(\text{C}_2\text{-C}_{10}$  alkenyl) naphthyl, phenyl  $(\text{C}_1\text{-C}_{10}$  alkyl), phenyl  $(\text{C}_2\text{-C}_{10}$  alkenyl), naphthyl  $(\text{C}_1\text{-C}_{10}$  alkyl), or naphthyl  $(\text{C}_2\text{-C}_{10}$  alkenyl);

$\text{R}^2$  being optionally substituted with  $\text{C}_1\text{-C}_4$  alkyl,  $\text{C}_2\text{-C}_4$  alkenyl,  $\text{C}_1\text{-C}_4$  alkoxy,  $-\text{OH}$ ,  $-\text{SH}$ ,  $-\text{CO}_2\text{R}^3$ ,  $\text{C}_3\text{-C}_{10}$  cycloalkyl,  $\text{C}_3\text{-C}_{10}$  cycloalkenyl, heterocycle having 3-10 ring atoms wherein the hetero atom is one or more of N, O, S, or any combination thereof, aryl,  $(\text{C}_1\text{-C}_{10}$  alk)aryl, ar $(\text{C}_1\text{-C}_{10}$  alkyl) or any combination thereof;

$\text{R}^2$  being optionally interrupted by oxygen, nitrogen, sulfur, or any combination thereof; and

$R^3$  is hydrogen,  $C_1$ - $C_4$  alkyl, or  $C_2$ - $C_4$  alkenyl.

The preferred naturally occurring amino acids for use in the present invention as amino acids or components of a peptide are alanine, arginine, asparagine, aspartic acid, citrulline, cysteine, cystine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, hydroxy proline,  $\gamma$ -carboxyglutamate, phenylglycine, or O-phosphoserine. The preferred amino acids are arginine, leucine, lysine, phenylalanine, tyrosine, tryptophan, valine, and phenylglycine.

The preferred non-naturally occurring amino acids for use in the present invention are  $\beta$ -alanine,  $\alpha$ -amino butyric acid,  $\gamma$ -amino butyric acid,  $\gamma$ -(aminophenyl) butyric acid,  $\alpha$ -amino isobutyric acid, citrulline,  $\epsilon$ -amino caproic acid, 7-amino heptanoic acid,  $\beta$ -aspartic acid, aminobenzoic acid, aminophenyl acetic acid, aminophenyl butyric acid,  $\gamma$ -glutamic acid, cysteine (ACM),  $\epsilon$ -lysine,  $\epsilon$ -lysine (A-Fmoc), methionine sulfone, norleucine, norvaline, ornithine, d-ornithine, p-nitro-phenylalanine, hydroxy proline, 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid, and thioproline.

Poly amino acids are either peptides or two or more amino acids linked by a bond formed by other groups which can be linked, *e.g.*, an ester, anhydride or an anhydride linkage. Special mention is made of non-naturally occurring poly amino acids and particularly non-naturally occurring hetero-poly amino acids, i.e. of mixed amino acids.

Peptides are two or more amino acids joined by a peptide bond. Peptides can vary in length from di-peptides with two amino acids to polypeptides with several hundred amino acids. See, Walker, Chambers Biological Dictionary, Cambridge, England: Chambers Cambridge, 1989, page 215. Special mention is made of non-naturally occurring peptides and particularly non-naturally occurring peptides of mixed amino acids. Special mention is also made of di-peptides tri-peptides, tetra-peptides, and penta-

peptides, and particularly, the preferred peptides are di-peptides and tri-peptides. Peptides can be homo- or hetero- peptides and can include natural amino acids, synthetic amino acids, or any combination thereof.

#### 5                    Proteinoid Complexing Perturbants

Proteinoids are artificial polymers of amino acids. The proteinoids preferably are prepared from mixtures of amino acids. Preferred proteinoids are condensation polymers, and most preferably, are thermal condensation polymers. These polymers may be directed or random polymers. Proteinoids can be linear,  
10    branched, or cyclical, and certain proteinoids can be units of other linear, branched, or cyclical proteinoids.

Special mention is made of diketopiperazines. Diketopiperazines are six member ring compounds. The ring includes two nitrogen atoms and is substituted at two carbons with two oxygen atoms. Preferably, the carbonyl  
15    groups are at the 1 and 4 ring positions. These rings can be optionally, and most often are, further substituted.

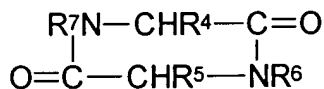
Diketopiperazine ring systems may be generated during thermal polymerization or condensation of amino acids or amino acid derivatives. (Gyore, J; Ecet M. *Proceedings Fourth ICTA (Thermal Analysis)*, 1974, 2, 387-394  
20    (1974)). These six membered ring systems were presumably generated by intramolecular cyclization of the dimer prior to further chain growth or directly from a linear peptide (Reddy, A.V., *Int. J. Peptide Protein Res.*, 40, 472-476 (1992); Mazurov, A.A. et al., *Int. J. Peptide Protein Res.*, 42, 14-19 (1993)).

Diketopiperazines can also be formed by cyclodimerization of amino  
25    acid ester derivatives as described by Katchalski et al., *J. Amer. Chem. Soc.*, 68, 879-880 (1946), by cyclization of dipeptide ester derivatives, or by thermal dehydration of amino acid derivatives and high boiling solvents as described by Kopple et al., *J. Org. Chem.*, 33 (2), 862-864 (1968).

In a typical synthesis of a diketopiperazine, the COOH group(s) of an amino acid benzyl ester are activated in a first step to yield a protected ester. The amine is deprotected and cyclized via dimerization in a second step, providing a diketopiperazine di-ester. Finally, the COOH group(s) are deprotected to provide the diketopiperazine.

Diketopiperazines typically are formed from  $\alpha$ -amino acids. Preferably, the  $\alpha$ -amino acids of which the diketopiperazines are derived are glutamic acid, aspartic acid, tyrosine, phenylalanine, and optical isomers of any of the foregoing.

Special mention is made of diketopiperazines of the formula



II

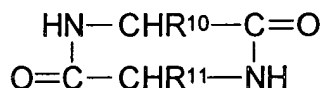
wherein  $\text{R}^4$ ,  $\text{R}^5$ ,  $\text{R}^6$ , and  $\text{R}^7$  independently are hydrogen,  $\text{C}_1$ - $\text{C}_{24}$  alkyl,  $\text{C}_1$ - $\text{C}_{24}$  alkenyl, phenyl, naphthyl, ( $\text{C}_1$ - $\text{C}_{10}$  alkyl)phenyl, ( $\text{C}_1$ - $\text{C}_{10}$  alkenyl)phenyl, ( $\text{C}_1$ - $\text{C}_{10}$  alkyl)naphthyl, ( $\text{C}_1$ - $\text{C}_{10}$  alkenyl)naphthyl, phenyl ( $\text{C}_1$ - $\text{C}_{10}$  alkyl), phenyl( $\text{C}_1$ - $\text{C}_{10}$  alkenyl), naphthyl ( $\text{C}_1$ - $\text{C}_{10}$  alkyl), and naphthyl ( $\text{C}_1$ - $\text{C}_{10}$  alkenyl); any of  $\text{R}^4$ ,  $\text{R}^5$ ,  $\text{R}^6$ , and  $\text{R}^7$  independently may optionally be substituted with  $\text{C}_1$ - $\text{C}_4$  alkyl,  $\text{C}_1$ - $\text{C}_4$  alkenyl,  $\text{C}_1$ - $\text{C}_4$  alkoxy, -OH, -SH, and  $-\text{CO}_2\text{R}^8$  or any combination thereof;  $\text{R}^8$  is hydrogen,  $\text{C}_1$ - $\text{C}_4$  alkyl or  $\text{C}_1$ - $\text{C}_4$  alkenyl; and any of  $\text{R}^4$ ,  $\text{R}^5$ ,  $\text{R}^6$ , and  $\text{R}^7$  independently may optionally be interrupted by oxygen, nitrogen, sulfur, or any combination thereof.

The phenyl or naphthyl groups may optionally be substituted. Suitable, but non-limiting, examples of substituents are  $\text{C}_1$ - $\text{C}_6$  alkyl,  $\text{C}_1$ - $\text{C}_6$  alkenyl,  $\text{C}_1$ - $\text{C}_6$  alkoxy, -OH, -SH, or  $\text{CO}_2\text{R}^9$  wherein  $\text{R}^9$  is hydrogen,  $\text{C}_1$ - $\text{C}_6$  alkyl, or  $\text{C}_1$ - $\text{C}_6$  alkenyl.

Preferably,  $R^6$  and  $R^7$  independently are hydrogen,  $C_1$ - $C_4$  alkyl or  $C_1$ - $C_4$  alkenyl. Special mention is made of diketopiperazines which are preferred complexing perturbants. These diketopiperazines include the unsubstituted diketopiperazine in which  $R^4$ ,  $R^5$ ,  $R^6$ , and  $R^7$  are hydrogen, and diketopiperazines  
 5 which are substituted at one or both of the nitrogen atoms in the ring, i.e. mono or di-N-substituted. Special mention is made of the N-substituted diketopiperazine wherein one or both of the nitrogen atoms is substituted with a methyl group.

Special mention is also made of diketopiperazines of the formula

10



III

wherein  $R^{10}$  and  $R^{11}$  independently are hydrogen,  $C_1$ - $C_{24}$  alkyl,  $C_1$ - $C_{24}$  alkenyl,  
 15 phenyl, naphthyl, ( $C_1$ - $C_{10}$  alkyl)phenyl, ( $C_1$ - $C_{10}$  alkenyl)phenyl, ( $C_1$ - $C_{10}$  alkyl)naphthyl, ( $C_1$ - $C_{10}$  alkenyl)naphthyl, phenyl ( $C_1$ - $C_{10}$  alkyl), phenyl( $C_1$ - $C_{10}$  alkenyl), naphthyl ( $C_1$ - $C_{10}$  alkyl), and naphthyl ( $C_1$ - $C_{10}$  alkenyl); but both  $R^{10}$  and  $R^{11}$  can not be hydrogen; either or both  $R^{10}$  or  $R^{11}$  independently may optionally be substituted with  $C_1$ - $C_4$  alkyl,  $C_1$ - $C_4$  alkenyl,  $C_1$ - $C_4$  alkoxy, -OH, -SH, and -  
 20  $\text{CO}_2\text{R}^{12}$  or any combination thereof;  $R^{12}$  is hydrogen,  $C_1$ - $C_4$  alkyl or  $C_1$ - $C_4$  alkenyl; and either or both  $R^{10}$  and  $R^{11}$  independently may optionally be interrupted by oxygen, nitrogen, sulfur, or any combination thereof.

The phenyl or naphthyl groups may optionally be substituted.

Suitable, but non-limiting, examples of substituents are  $C_1$ - $C_6$  alkyl,  $C_1$ - $C_6$  alkenyl,  
 25  $C_1$ - $C_6$  alkoxy, -OH, -SH, or  $\text{CO}_2\text{R}^{13}$  wherein  $R^{13}$  is hydrogen,  $C_1$ - $C_6$  alkyl, or  $C_1$ - $C_6$  alkenyl. When one of  $R^{10}$  or  $R^{11}$  is hydrogen, the diketopiperazine is mono-carbon-(C)-substituted. When neither  $R^{10}$  nor  $R^{11}$  is hydrogen, the diketopiperazine is di-carbon-(C)-substituted.

Preferably,  $R^{10}$ ,  $R^{11}$ , or both  $R^{10}$  and  $R^{11}$ , contain at least one  
 30 functional group, a functional group being a non-hydrocarbon portion responsible

for characteristic reactions of the molecule. Simple functional groups are heteroatoms including, but not limited to halogens, oxygen, sulfur, nitrogen, and the like, attached to, the carbon of an alkyl group by a single or multiple bond. Other functional groups include, but are not limited to, for example, hydroxyl groups, carboxyl groups, amide groups, amine groups, substituted amine groups, and the like.

Preferred diketopiperazines are those which are substituted at one or two of the carbons of the ring with a functional group that includes at least one carboxyl functionality.

#### Amino Acid(s)/Diketopiperazine Complexing Perturbants

Diketopiperazines may also be polymerized with additional amino acids to form constructs of at least one amino acid or an ester or an amide thereof and at least one diketopiperazine, preferably covalently bonded to one another.

When the diketopiperazine is polymerized with additional amino acids, one or more of the R groups must contain at least one functional group, a functional group being a non-hydrocarbon portion responsible for characteristic reactions of the molecule. Simple functional groups are heteroatoms including, but not limited to halogens, oxygen, sulfur, nitrogen, and the like, attached to, the carbon of an alkyl group by a single or multiple bond. Other functional groups include, but are not limited to, for example, hydroxyl groups, carboxyl groups, amide groups, amine groups, substituted amine groups, and the like.

Special mention is also made of diketopiperazines which are preferred components of the amino acids/diketopiperazine perturbants of the present invention. Such preferred diketopiperazines are those which are substituted at one or two of the carbons of the ring and preferably are substituted with a functional group that includes at least one carboxyl functionality.



Most preferably, the diketopiperazines in the amino acids/diketopiperazine perturbants are prepared from trifunctional amino acids such as L-glutamic acid and L-aspartic acid which cyclize to form diketopiperazines.

5           The diketopiperazines can generate a bis-carboxylic acid platform which can be further condensed with other amino acids to form the perturbant. Typically, the diketopiperazine will react and covalently bond with one or more of the amino acids through the functional group(s) of the R groups of the diketopiperazines. These unique systems, because of the cis-geometry imparted  
10 by the chiral components of the diketopiperazine ring (Lannom, H.K. et al., *Int. J. Peptide Protein Res.*, 28, 67-78 (1986)), provide an opportunity to systematically alter the structure of the terminal amino acids while holding the orientation between them fixed relative to non-cyclic analogs (Fusaoka et al., *Int. J. Peptide Protein Res.*, 34, 104-110 (1989); Ogura, H. et al., *Chem. Pharma.*  
15 *Bull.*, 23, 2474-2477 (1975). See also, Lee, B.H. et al. *J. Org. Chem.*, 49, 2418-2423 (1984); Buyle, R., *Helv. Chim. Acta*, 49, 1425, 1429 (1966). Other methods of polymerization known to those skilled in the art may lend themselves to amino acid/diketopiperazine polymerization as well.

20           The amino acids/diketopiperazine perturbants may include one or more of the same or different amino acids as well as one or more of the same or different diketopiperazines as described above.

Ester and amide derivatives of these amino acids/diketopiperazine perturbants are also useful in the present invention.

## 25           Modified Amino Acid Complexing Perturbants

Modified amino acids, poly amino acids or peptides are either acylated or sulfonated and include amino acid amides and sulfonamides.

Modified amino acids are typically prepared by modifying the amino acid or an ester thereof. Many of these compounds are prepared by acylation or sulfonation with agents having the formula



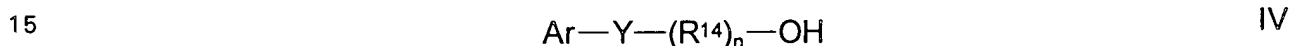
- 5 wherein:  $R^4$  is the appropriate radical to yield the modification indicated in the final product,



- 10 Y is C or  $\text{SO}_2$ , and X is a leaving group. Typical leaving groups include, but are not limited to, halogens such as, for example, chlorine, bromine, and iodine. Additionally, the corresponding anhydrides are modifying agents.

#### Acylated Amino Acid Complexing Perturbants

Special mention is made of acylated amino acids having the formula



wherein Ar is a substituted or unsubstituted phenyl or naphthyl;

- 20 Y is  $\begin{array}{c} \text{O} \\ || \\ -\text{C}- \end{array}$ ,  $\text{R}^{14}$  has the formula  $-\text{N}(\text{R}^{16}) - \text{R}^{15} - \begin{array}{c} \text{O} \\ || \\ -\text{C}- \end{array}$ , wherein:

$\text{R}^{15}$  is  $\text{C}_1$  to  $\text{C}_{24}$  alkyl,  $\text{C}_1$  to  $\text{C}_{24}$  alkenyl, phenyl, naphthyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) phenyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl) phenyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) naphthyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl) naphthyl, phenyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl), phenyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl), naphthyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) and naphthyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl);

- 25  $\text{R}^{15}$  is optionally substituted with  $\text{C}_1$  to  $\text{C}_4$  alkyl,  $\text{C}_1$  to  $\text{C}_4$  alkenyl,  $\text{C}_1$  to  $\text{C}_4$  alkoxy, -OH, -SH and  $-\text{CO}_2\text{R}^5$ , cycloalkyl, cycloalkenyl, heterocyclic alkyl, alkaryl, heteroaryl, heteroalkaryl, or any combination thereof;

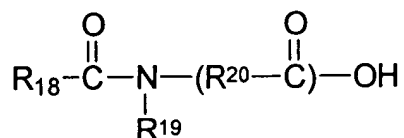
$\text{R}^{17}$  is hydrogen,  $\text{C}_1$  to  $\text{C}_4$  alkyl or  $\text{C}_1$  to  $\text{C}_4$  alkenyl;

- 30  $\text{R}^{15}$  is optionally interrupted by oxygen, nitrogen, sulfur or any combination thereof; and

$R^{16}$  is hydrogen,  $C_1$  to  $C_4$  alkyl or  $C_1$  to  $C_4$  alkenyl.

Special mention is also made of those having the formula

5



V

wherein:  $R^{18}$  is (i)  $C_3$ - $C_{10}$  cycloalkyl, optionally substituted with  $C_1$ - $C_7$  alkyl,

$C_2$ - $C_7$  alkenyl,  $C_1$ - $C_7$  alkoxy, hydroxy, phenyl, phenoxy or  $-CO_2R^{21}$ ,

10

wherein  $R^1$  is hydrogen,  $C_1$ - $C_4$  alkyl, or  $C_2$ - $C_4$  alkenyl; or

(ii)  $C_1$ - $C_6$  alkyl substituted with  $C_3$ - $C_{10}$  cycloalkyl;

$R^{19}$  is hydrogen,  $C_1$ - $C_4$  alkyl, or  $C_2$ - $C_4$  alkenyl;

$R^{20}$  is  $C_1$ - $C_{24}$  alkyl,  $C_2$ - $C_{24}$  alkenyl,  $C_3$ - $C_{10}$  cycloalkyl,  $C_3$ - $C_{10}$

cycloalkenyl, phenyl, naphthyl, ( $C_1$ - $C_{10}$  alkyl) phenyl, ( $C_2$ - $C_{10}$  alkenyl) phenyl, ( $C_1$ -

15

$C_{10}$  alkyl) naphthyl, ( $C_2$ - $C_{10}$  alkenyl) naphthyl, phenyl ( $C_1$ - $C_{10}$  alkyl), phenyl ( $C_2$ - $C_{10}$  alkenyl), naphthyl ( $C_1$ - $C_{10}$  alkyl) or naphthyl ( $C_2$ - $C_{10}$  alkenyl);

$R^{20}$  being optionally substituted with  $C_1$ - $C_4$  alkyl,  $C_2$ - $C_4$  alkenyl,  $C_1$ -

$C_4$  alkoxy,  $-OH$ ,  $-SH$ ,  $-CO_2R^{22}$ ,  $C_3$ - $C_{10}$  cycloalkyl,  $C_3$ - $C_{10}$  cycloalkenyl, heterocycle

having 3-10 ring atoms wherein the hetero atom is one or more of N, O, S or any

20

combination thereof, aryl, ( $C_1$ - $C_{10}$  alk)aryl, ar( $C_1$ - $C_{10}$  alkyl), or any combination thereof;

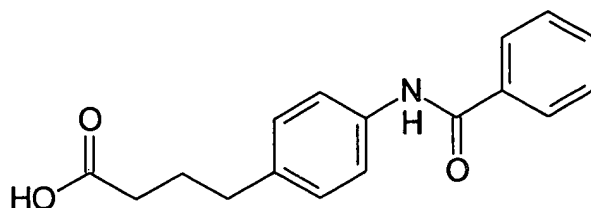
$R^{20}$  being optionally interrupted by oxygen, nitrogen, sulfur, or any

combination thereof; and

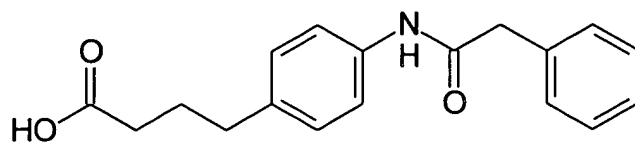
$R^{22}$  is hydrogen,  $C_1$ - $C_4$  alkyl, or  $C_2$ - $C_4$  alkenyl.

25

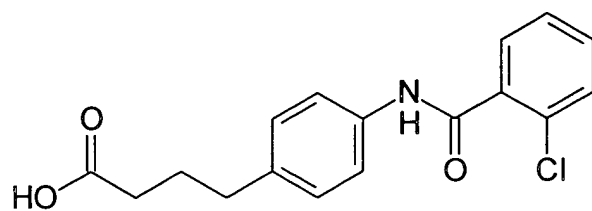
Some preferred acylated amino acids include salicyloyl phenylalanine, and the compounds having the formulas:



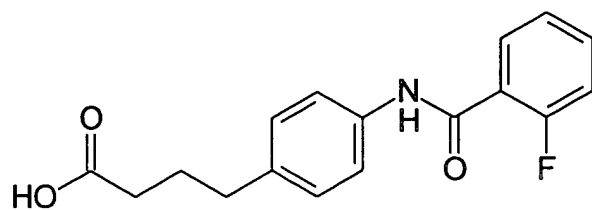
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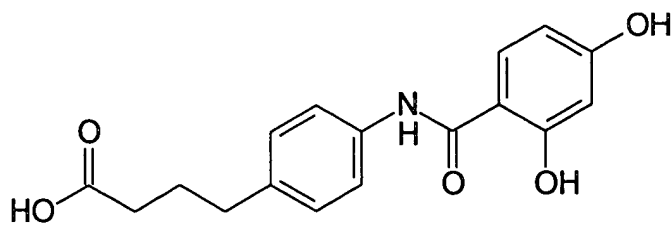
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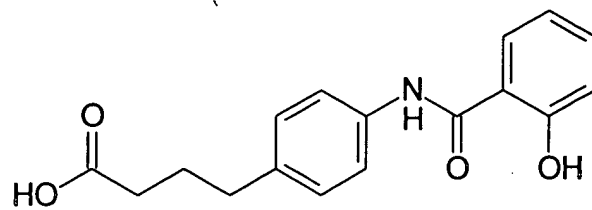
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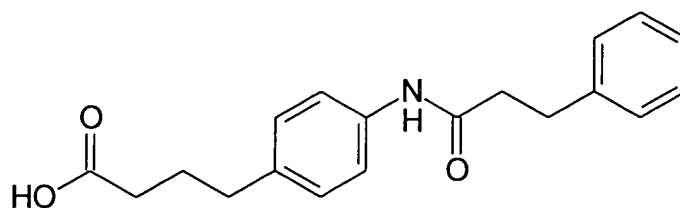
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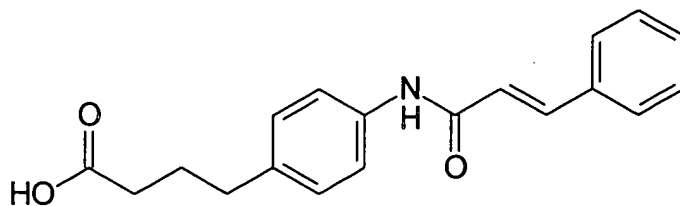
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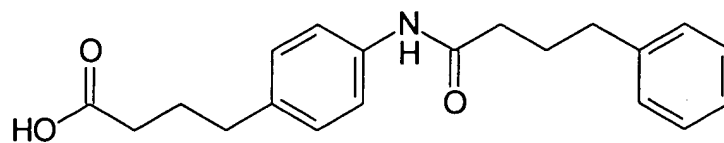
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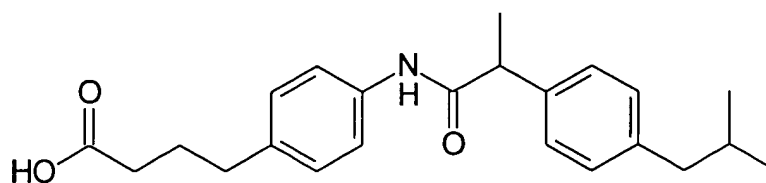
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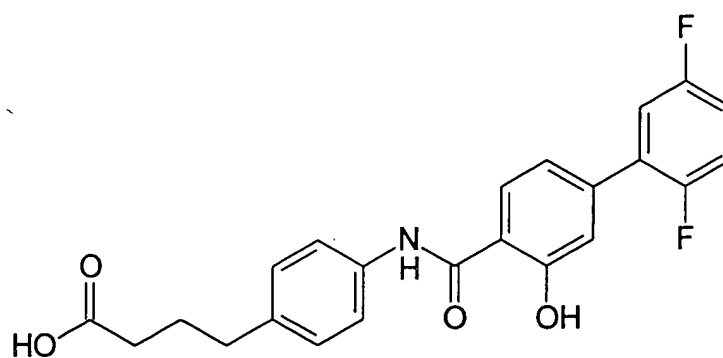
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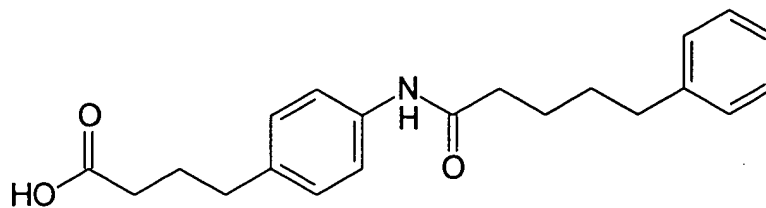
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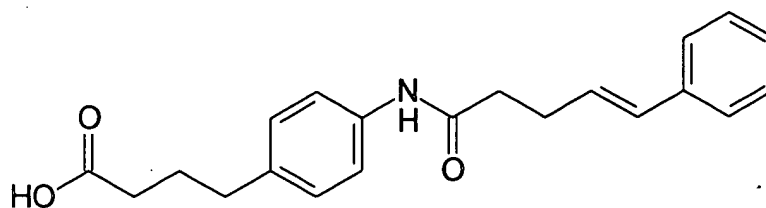
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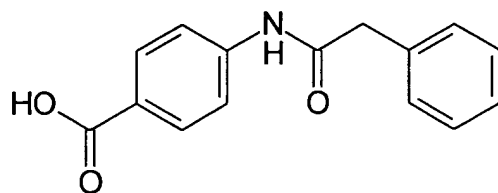
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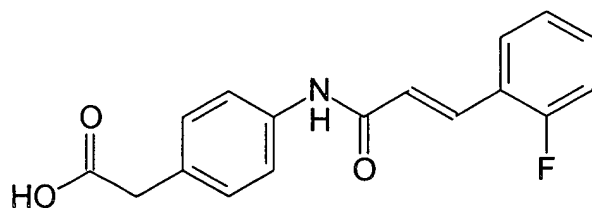
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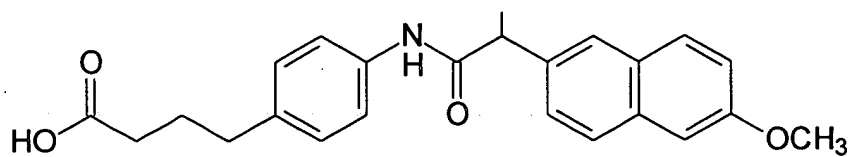
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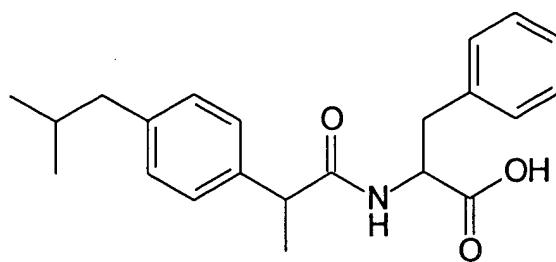
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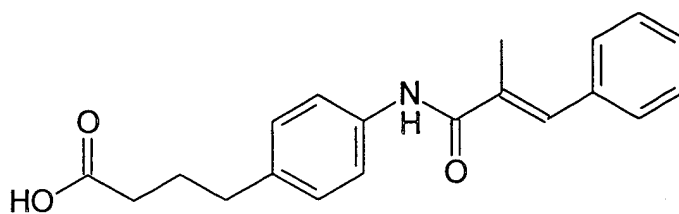
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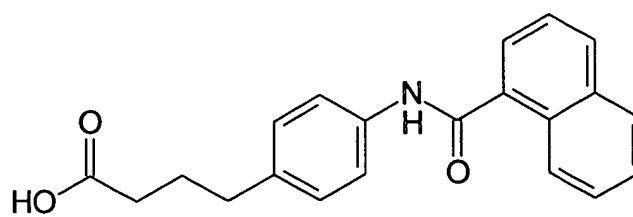
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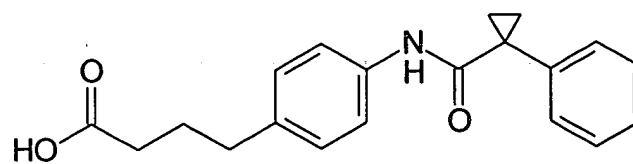
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XXIII

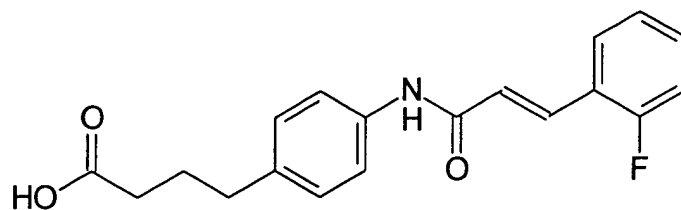


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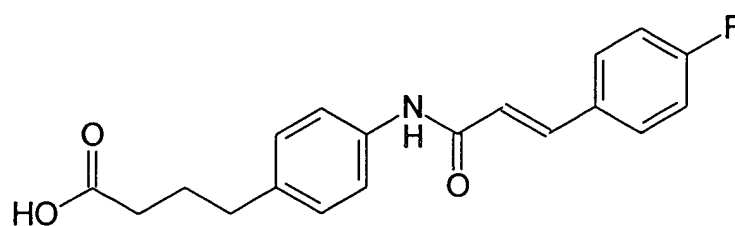


XXV

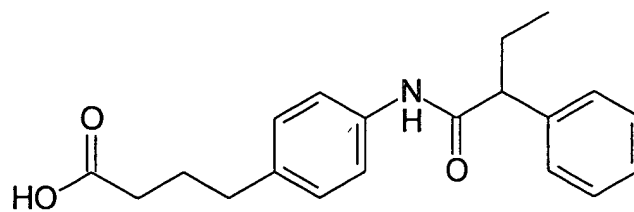




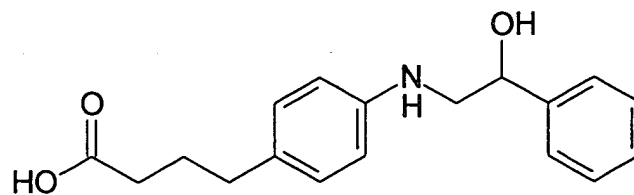
XXVI



XXVII

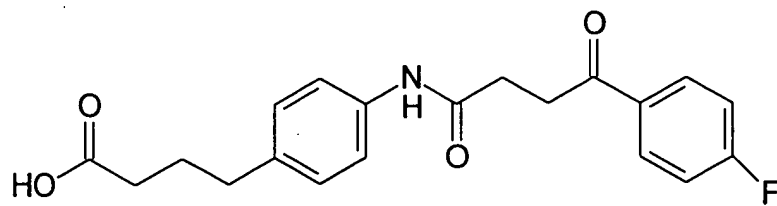


XXVIII

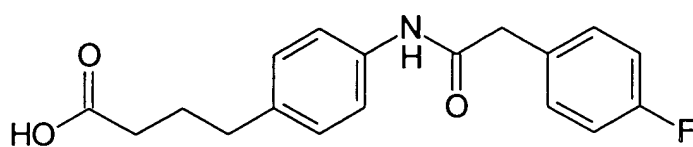


XXIX

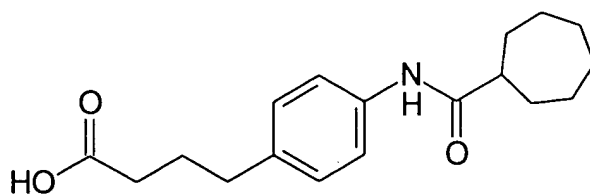
34



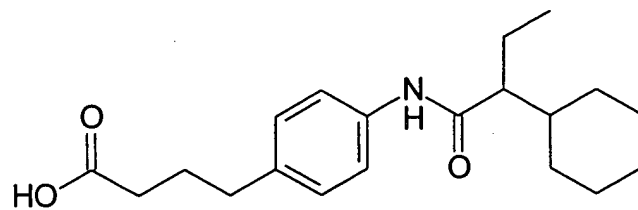
XXX



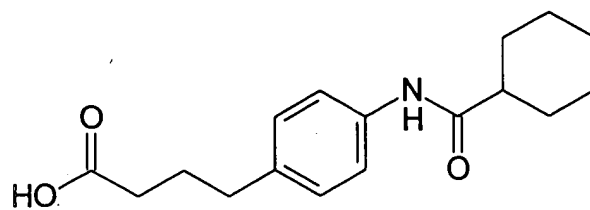
XXXI



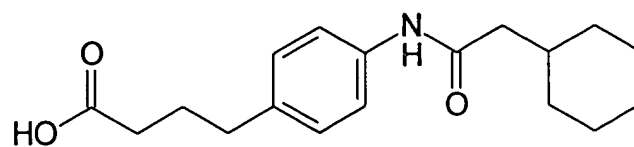
XXXII



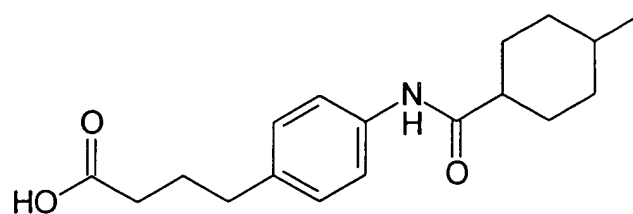
XXXIII



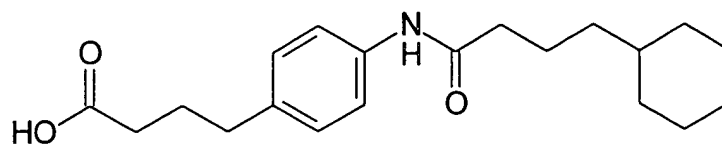
XXXIV



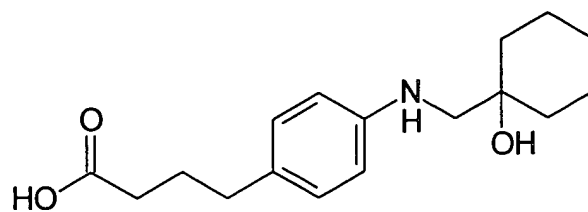
XXXV



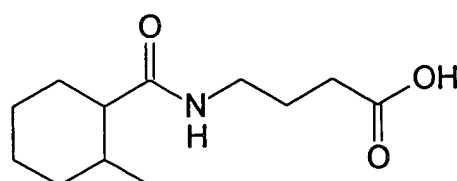
XXXVI



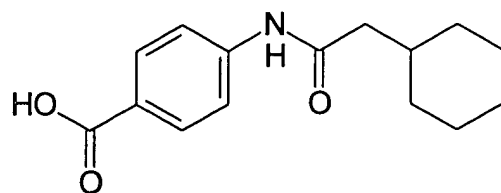
XXXVII



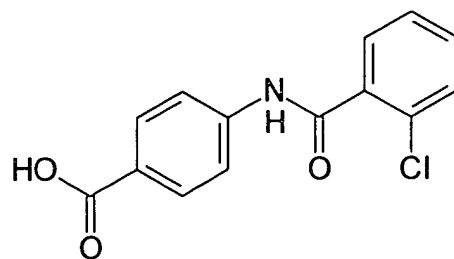
XXXVIII



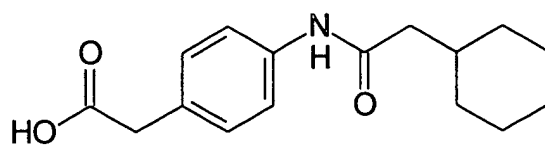
XXXIX



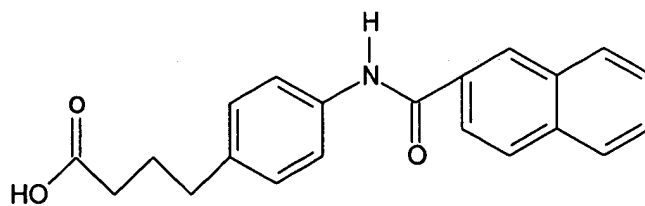
XL



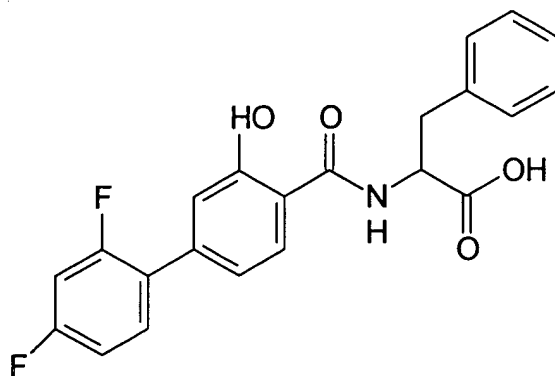
XLI



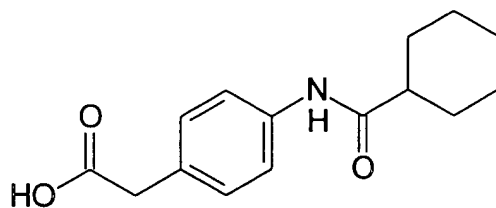
XLII



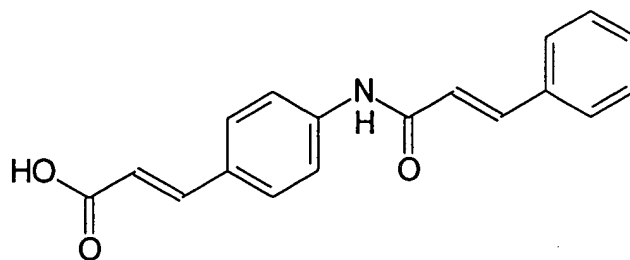
XLIIA



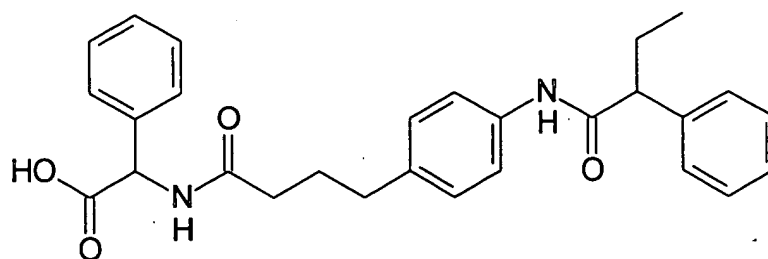
XLIII



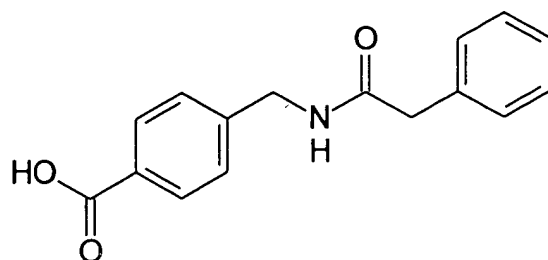
XLIV



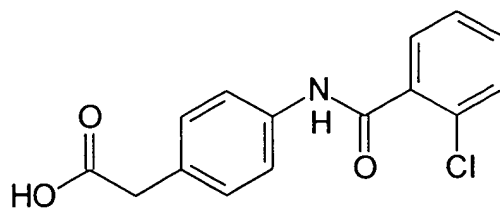
XLV



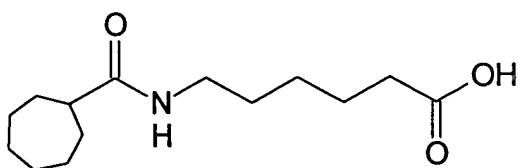
XLVI



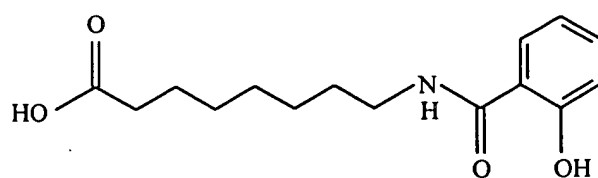
XLVII



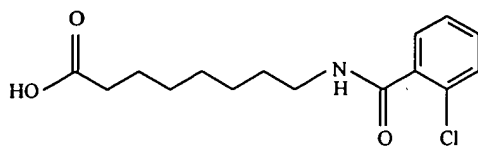
XLVIII



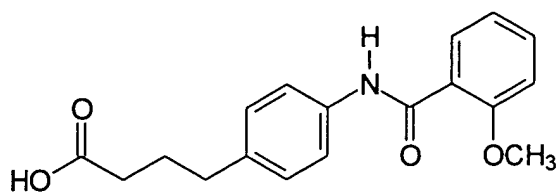
XLIX



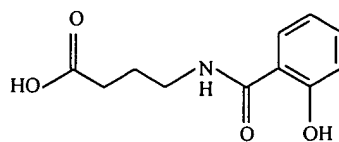
L



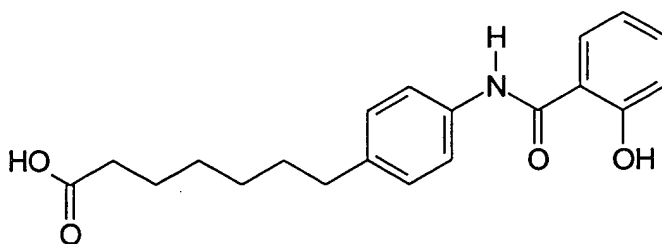
LI



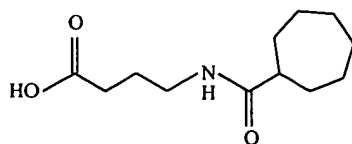
LII



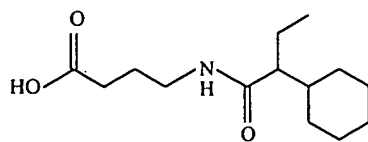
LIII



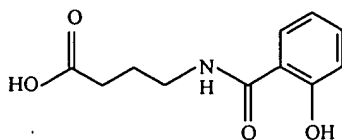
LIV



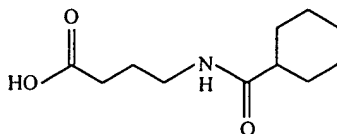
II-1



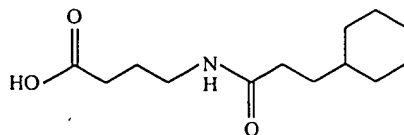
III-1



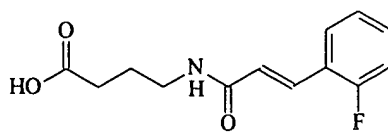
IV-1



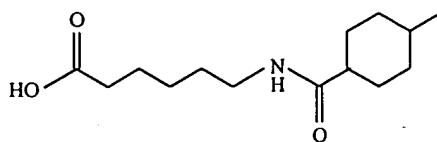
V-1



VI-1



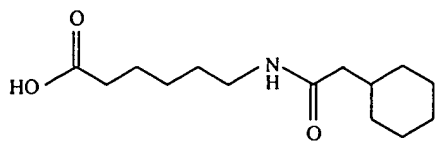
VII-1



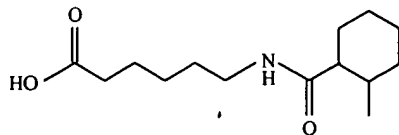
VIII-1



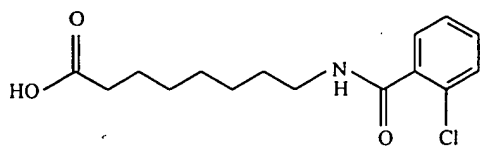
41



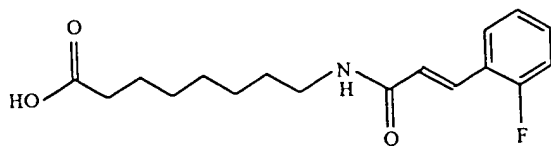
IX-1



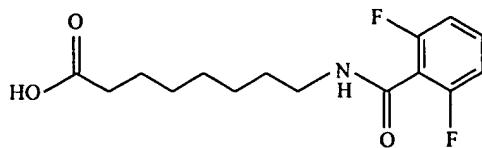
X-1



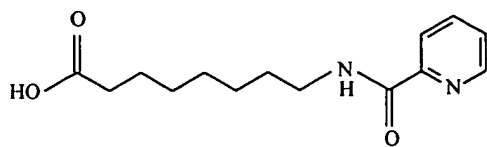
XI-1



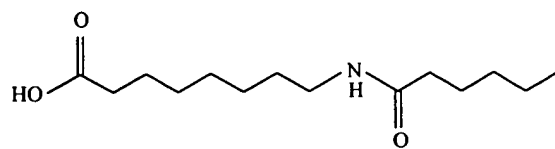
XII-



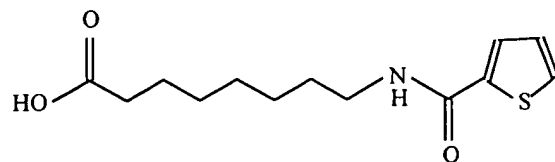
XIII-1



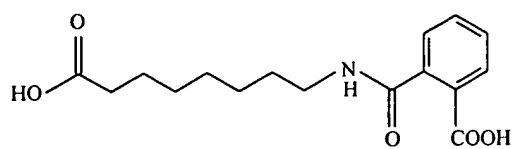
XIV-1



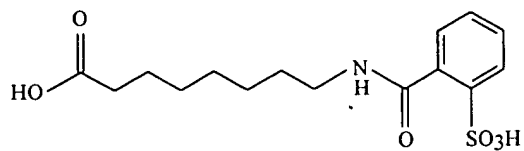
XV-1



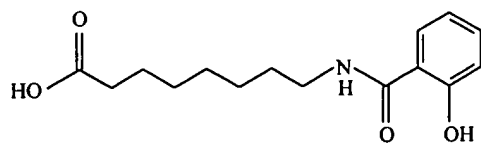
XVI-1



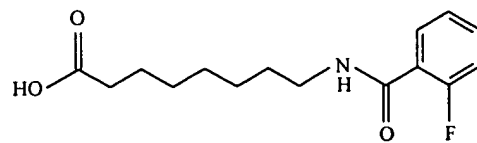
XVII-1



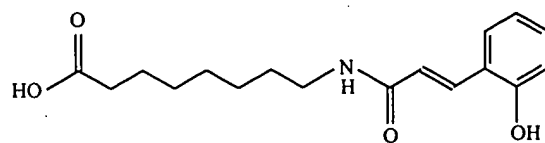
XVIII-1



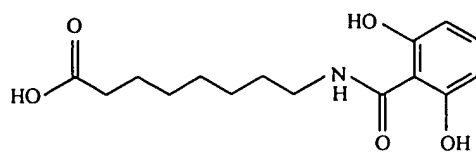
XIX-1



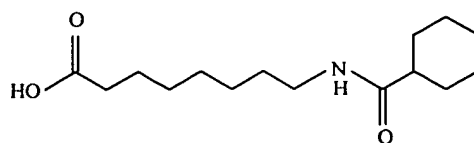
XX-1



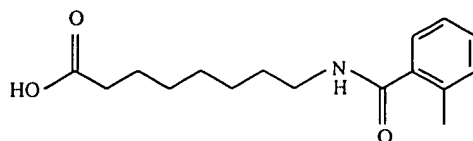
XXI-1



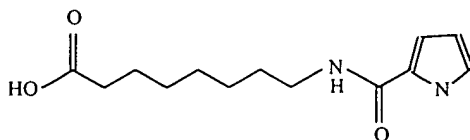
XXII-1



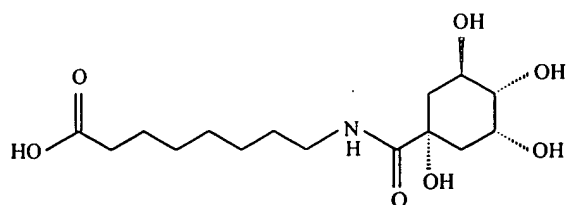
XXIII-1



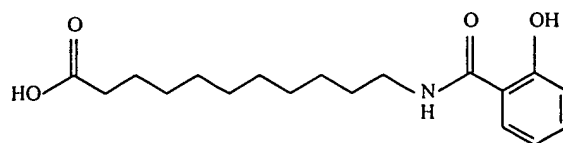
XXIV-1



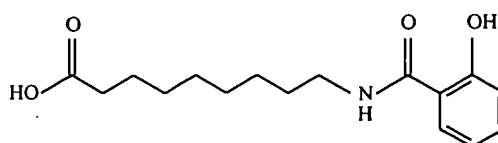
XXV-1



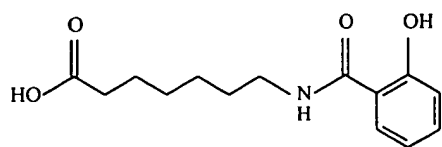
XXVI-1



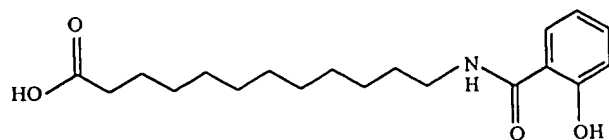
XXVII-1



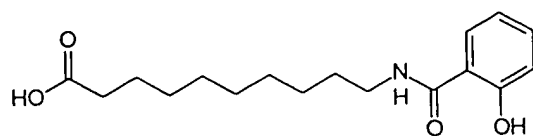
XXVII-1



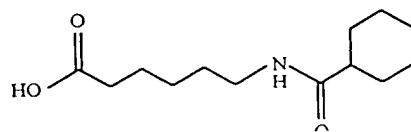
XXIX-1



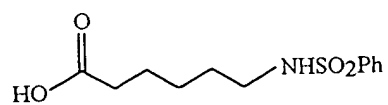
XXX-1



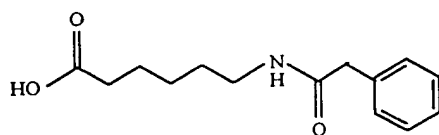
XXXI-1



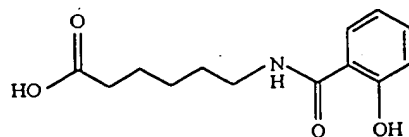
XXXII-1



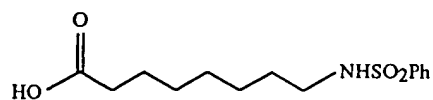
XXXIII-1



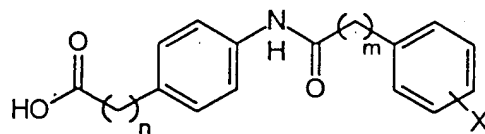
XXXIV-1



XXXV-1



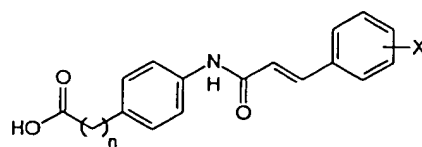
XXXVI-1



A

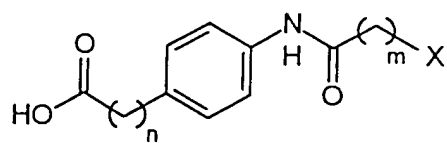
Compound	n	m	X
XXXVIIA	0	0	4-Cl
XXXVIII A	3	0	H
XXIXA	3	1	4-CH <sub>3</sub>
XL A	3	1	2-F
XL I A	3	1	2-CH <sub>3</sub>
XL II A	3	0	3-CF <sub>3</sub>
XL III A	3	4	H
XL IV A	3	0	3-Cl
XL V A	3	0	3-F
XL VI A	3	0	3-CH <sub>3</sub>
XL VII A	0	0	2-CF <sub>3</sub>
XL VIII A	1	2	H
XL IX A	3	2	2-F
LA	3	0	3,4-OCH <sub>2</sub> O-
L I A	3	0	2-COOH
L II A	1	0	2-OH
L III A	3	0	2,6-dihydroxy
L IV A	2	0	2-OH
L V A	0	0	2,4-difluoro
L VI A	2	0	2,6-dihydroxy

LVIIA	0	0	4-CF <sub>3</sub>
LVIIIA	3	0	3-NMe <sub>2</sub>
LIXA	2	0	3-NMe <sub>2</sub>
LXA	3	0	2,6-dimethyl
LXIA	3	0	2-NO <sub>2</sub>
LXIIA	3	0	2-CF <sub>3</sub>
LXIIIA	3	0	4- <i>n</i> -Pr
LXIVA	3	0	2-NH <sub>2</sub>
LXVA	3	0	2-OCH <sub>3</sub>
LXVIA	3	0	3-NO <sub>2</sub>
LXVIIA	3	0	3-NH <sub>2</sub>
LXVIIIA	2	0	2-NO <sub>2</sub>
LXIXA	2	0	2-NH <sub>2</sub>
LXXA	3	0	2-OCF <sub>3</sub>
LXXIA	2	0	2-OCH <sub>3</sub>
LXXIIA	2	0	2-OCF <sub>3</sub>



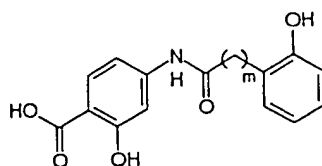
B

Compound	n	X
LXXIIIB	3	4-CF <sub>3</sub>
LXXIVB	1	2-F
LXXVB	1	4-CF <sub>3</sub>
LXXVIB	3	3,4-dimethoxy
LXXVIIIB	0	3-OCH <sub>3</sub>
LXXVIIIIB	3	3-OCH <sub>3</sub>
LXXIXB	3	2,6-difluoro
LXXXB	3	4-CH <sub>3</sub>
LXXXIB	1	4-OCH <sub>3</sub>
LXXXIIB	2	2-F
LXXXIIIB	0	2-F
LXXXIVB	2	4-OCH <sub>3</sub>
LXXXVB	0	2-OCH <sub>3</sub>
LXXXVIB	2	2-OCH <sub>3</sub>
LXXXVIIIB	0	4-CF <sub>3</sub>
LXXXVIIIIB	3	3-F
LXXXIXB	3	2-OCH <sub>3</sub>



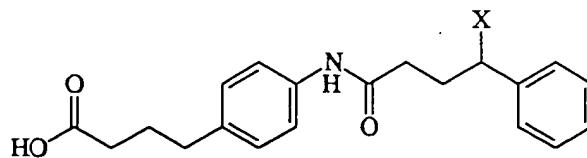
C

Compound	n	m	X
VII-C	3	0	2-carboxycyclohexyl
VIII-C	3	3	cyclohexyl
IX-C	3	0	2-adamantyl
X-C	3	0	1-morpholino



D

Compound	m
XI-D	0
XII-D	3



E

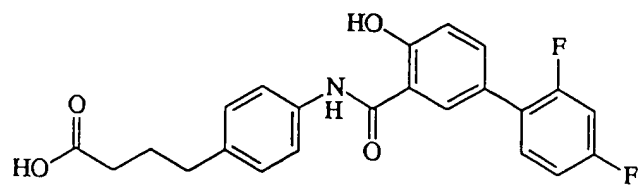
Compound	X
XIII-E	OH
XIV-E	=O



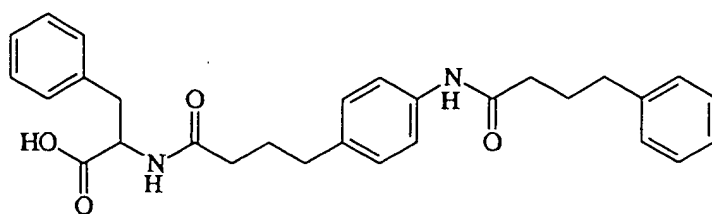


Compound	n
XV-F	0
XVI-F	2

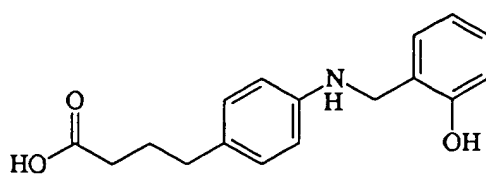
[illegible]



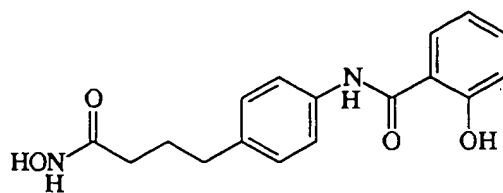
CII-1



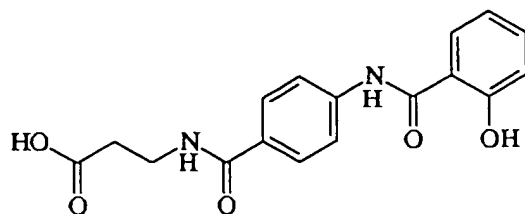
CIII-1



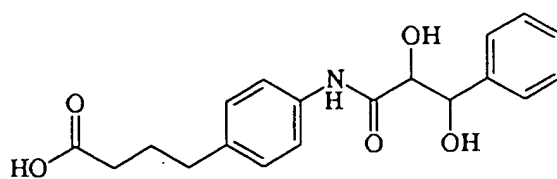
CIV-1



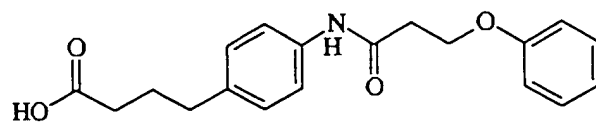
CV-1



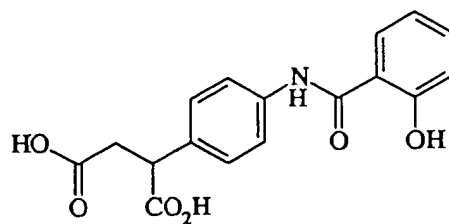
CVI-1



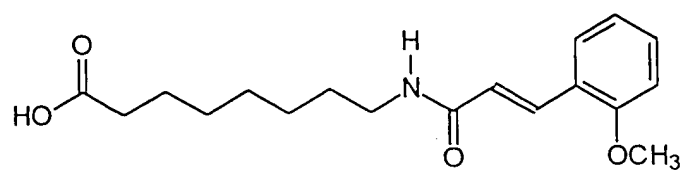
CVII-1



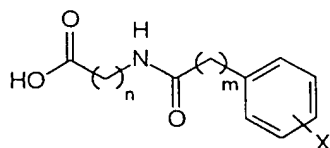
CVIII-1



CIX-1

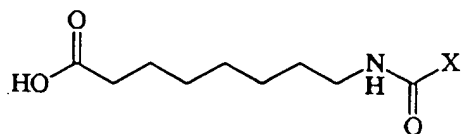


CX-1



G

Compound	n	m	X
CXI-G	6	0	2-OH
CXII-G	7	3	H
CXIII-G	7	0	2-I
CXIV-G	7	0	2-Br
CXV-G	7	0	3-NO <sub>2</sub>
CXVI-G	7	0	3-N(CH <sub>3</sub> ) <sub>2</sub>
CXVII-G	7	0	2-NO <sub>2</sub>
CXVIII-G	7	0	4-NO <sub>2</sub>
CXIX-G	9	0	2-OH



H

Compound	X
CXX-H	1-morpholino
CXXI-H	O- <i>t</i> -Butyl
CXXII-H	CH(CH <sub>2</sub> Ph)NC(O)O- <i>t</i> -Bu
CXXIII-H	2-hydroxyphenyl

organic acid compounds, and their salts, having an aromatic amide group, having a hydroxy group substituted in the ortho position on the aromatic ring, and a lipophilic chain with from about 4 carbon atoms to about 20 atoms in the chain are also useful as perturbants. In a preferred form the lipophilic chain can have from 5 to 20 carbon atoms.

Perturbants which are also useful also include those having the formula



wherein Ar is a substituted or unsubstituted phenyl or naphthyl;

R<sup>7</sup> is selected from the group consisting of C<sub>4</sub> to C<sub>20</sub> alkyl, C<sub>4</sub> to C<sub>20</sub> alkenyl, phenyl, naphthyl, (C<sub>1</sub> to C<sub>10</sub> alkyl) phenyl, (C<sub>1</sub> to C<sub>10</sub> alkenyl) phenyl, (C<sub>1</sub> to C<sub>10</sub> alkyl) naphthyl, (C<sub>1</sub> to C<sub>10</sub> alkenyl) naphthyl, phenyl (C<sub>1</sub> to C<sub>10</sub> alkyl), phenyl (C<sub>1</sub> to C<sub>10</sub> alkenyl), naphthyl (C<sub>1</sub> to C<sub>10</sub> alkyl), and naphthyl (C<sub>1</sub> to C<sub>10</sub> alkenyl);

R<sup>8</sup> is selected from the group consisting of hydrogen, C<sub>1</sub> to C<sub>4</sub> alkyl, C<sub>1</sub> to C<sub>4</sub> alkenyl, hydroxy, and C<sub>1</sub> to C<sub>4</sub> alkoxy;

$R^7$  is optionally substituted with  $C_1$  to  $C_4$  alkyl,  $C_1$  to  $C_4$  alkenyl,  $C_1$  to  $C_4$  alkoxy, -OH, -SH and  $-CO_2R^9$  or any combination thereof;

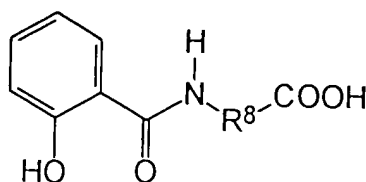
$R^9$  is hydrogen,  $C_1$  to  $C_4$  alkyl or  $C_1$  to  $C_4$  alkenyl;

$R^7$  is optionally interrupted by oxygen, nitrogen, sulfur or any combination thereof;

with the proviso that the compounds are not substituted with an amino group in the position alpha to the acid group, or salts thereof.

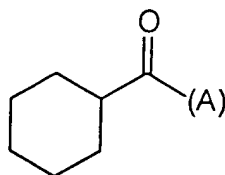
The preferred  $R^6$  groups are of  $C_4$  to  $C_{20}$  alkyl and  $C_4$  to  $C_{20}$  alkenyl. The most preferred  $R^6$  groups are  $C_5$  to  $C_{20}$  alkyl and  $C_5$  to  $C_{20}$  alkenyl.

A preferred carrier compound can have the formula



wherein  $R^7$  is defined above.

Special mention is made of compounds having the formula:



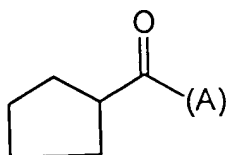
LV

wherein A is Try, Leu, Arg, Trp, or Cit; and optionally wherein if A is Try, Arg, Trp or Cit; A is acylated at 2 or more functional groups.

Preferred compounds also include those wherein A is Try; A is Tyr and is acylated at 2 functional groups; A is Leu; A is Arg; A is Arg and is

acylated at 2 functional groups; A is Trp; A is Trp and is acylated at 2 functional groups; A is Cit; and A is Cit and is acylated at 2 functional groups.

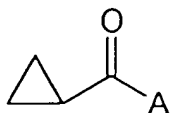
Special mention is also made of compounds having the formula:



LVI

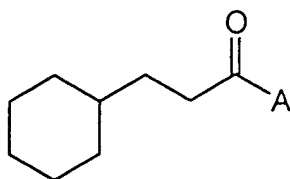
wherein A is Arg or Leu; and

wherein if A is Arg, A is optionally acylated at 2 or more functional groups;



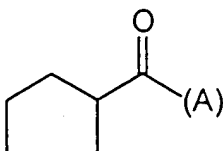
LVII

where A is Leu or phenylglycine;



LVIII

wherein A is phenylglycine; and



LIX

wherein A is phenylglycine.

Acylated amino acids may be prepared by reacting single amino acids, mixtures of two or more amino acids, or amino acid esters with an amine modifying agent which reacts with free amino moieties present in the amino acids to form amides.

Suitable, but non-limiting, examples of acylating agents useful in preparing acylated amino acids include

acid chloride acylating agents having the formula  $R^{23}-\overset{\overset{O}{\parallel}}{C}-X$  wherein:

$R^{23}$  an appropriate group for the modified amino acid being prepared, such as, but not limited to, alkyl, alkenyl, cycloalkyl, or aromatic, and particularly methyl, ethyl, cyclohexyl, cyclophenyl, phenyl, or benzyl, and X is a leaving group. Typical leaving groups include, but are not limited to, halogens such as chlorine, bromine and iodine.

Examples of the acylating agents include, but are not limited to, acyl halides including, but not limited to, acetyl chloride, propyl chloride, cyclohexanoyl chloride, cyclopentanoyl chloride, and cycloheptanoyl chloride, benzoyl chloride, hippuryl chloride and the like; and anhydrides, such as acetic anhydride, propyl anhydride, cyclohexanoic anhydride, benzoic anhydride, hippuric anhydride and the like. Preferred acylating agents include benzoyl chloride, hippuryl chloride, acetyl chloride, cyclohexanoyl chloride, cyclopentanoyl chloride, and cycloheptanoyl chloride.

The amine groups can also be modified by the reaction of a carboxylic acid with coupling agents such as the carbodiimide derivatives of amino acids, particularly hydrophilic amino acids such as phenylalanine, tryptophan, and tyrosine. Further examples include dicyclohexylcarbodiimide and the like.



If the amino acid is multifunctional, i.e. has more than one  $-OH$ ,  $-NH_2$  or  $-SH$  group, then it may optionally be acylated at one or more functional groups to form, for example, an ester, amide, or thioester linkage.

For example, in the preparation of many acylated amino acids, the amino acids are dissolved in an aqueous alkaline solution of a metal hydroxide, *e.g.*, sodium or potassium hydroxide and the acylating agent added. The reaction time can range from about 1 hour and about 4 hours, preferably about 2-2.5 hours. The temperature of the mixture is maintained at a temperature generally ranging between about  $5^{\circ}C$  and about  $70^{\circ}C$ , preferably between about  $10^{\circ}C$  and about  $50^{\circ}C$ . The amount of alkali employed per equivalent of  $NH_2$  groups in the amino acids generally ranges between about 1.25 moles and about 3 moles, and is preferably between about 1.5 moles and about 2.25 moles per equivalent of  $NH_2$ . The pH of the reaction solution generally ranges between about pH 8 and about pH 13, and is preferably between about pH 10 and about pH 12. The amount of amino modifying agent employed in relation to the quantity of amino acids is based on the moles of total free  $NH_2$  in the amino acids. In general, the amino modifying agent is employed in an amount ranging between about 0.5 and about 2.5 mole equivalents, preferably between about 0.75 and about 1.25 equivalents, per molar equivalent of total  $NH_2$  groups in the amino acids.

The modified amino acid formation reaction is quenched by adjusting the pH of the mixture with a suitable acid, *e.g.*, concentrated hydrochloric acid, until the pH reaches between about 2 and about 3. The mixture separates on standing at room temperature to form a transparent upper layer and a white or off-white precipitate. The upper layer is discarded, and modified amino acids are collected by filtration or decantation. The crude modified amino acids are then mixed with water. Insoluble materials are removed by filtration and the filtrate is dried in vacuo. The yield of modified amino acids generally ranges between about 30 and about 60%, and usually

about 45%. The present invention also contemplates amino acids which have been modified by multiple acylation, *e.g.*, diacylation or triacylation.

If desired, amino acid esters, such as, for example benzyl, methyl, or ethyl esters of amino acid compounds, may be used to prepare the modified amino acids of the invention. The amino acid ester, dissolved in a suitable organic solvent such as dimethylformamide, pyridine, or tetrahydrofuran is reacted with the appropriate amino modifying agent at a temperature ranging between 5°C and about 70°C, preferably about 25°C, for a period ranging between about 7 and about 24 hours. The amount of amino acid modifying agent used relative to the amino acid ester is the same as described above for amino acids. This reaction may be carried out with or without a base such as, for example, triethylamine or diisopropylethylamine.

If amino acid esters or amides are the starting materials, they are dissolved in a suitable organic solvent such as dimethylformamide or pyridine, are reacted with the amino modifying agent at a temperature ranging between about 5°C and about 70°C, preferably about 25°C, for a period ranging between about 7 and about 24 hours. The amount of amino modifying agents used relative to the amino acid esters are the same as described above for amino acids.

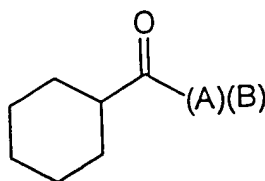
Thereafter, the reaction solvent is removed under negative pressure and optionally the ester or amide functionality can be removed by hydrolyzing the modified amino acid ester with a suitable alkaline solution, *e.g.*, 1N sodium hydroxide, at a temperature ranging between about 50°C and about 80°C, preferably about 70°C, for a period of time sufficient to hydrolyze off the ester group and form the modified amino acid having a free carboxyl group. The hydrolysis mixture is then cooled to room temperature and acidified, *e.g.*, with an aqueous 25% hydrochloric acid solution, to a pH ranging between about 2 and about 2.5. The modified amino acid precipitates out of solution and is recovered by conventional means such as filtration or decantation.

The modified amino acids may be purified by acid precipitation, recrystallization or by fractionation on solid column supports. Fractionation may be performed on a suitable solid column supports such as silica gel, alumina, using solvent mixtures such as acetic acid/butanol/water as the mobile phase; reverse phase column supports using trifluoroacetic acid/acetonitrile mixtures as the mobile phase; and ion exchange chromatography using water as the mobile phase. The modified amino acids may also be purified by extraction with a lower alcohol such as methanol, butanol, or isopropanol to remove impurities such as inorganic salts.

The modified amino acids generally are soluble in alkaline aqueous solution ( $\text{pH} \geq 9.0$ ); partially soluble in ethanol, n-butanol and 1:1 (v/v) toluene/ethanol solution and insoluble in neutral water. The alkali metal salts, *e.g.*, the sodium salt of the derivatized amino acids are generally soluble in water at about a pH of 6-8.

In poly amino acids or peptides, one or more of the amino acids may be modified (acylated). Modified poly amino acids and peptides may include one or more acylated amino acid(s). Although linear modified poly amino acids and peptides will generally include only one acylated amino acid, other poly amino acid and peptide configurations can include more than one acylated amino acid. Poly amino acids and peptides can be polymerized with the acylated amino acid(s) or can be acylated after polymerization.

Special mention is made of the compound:



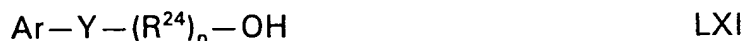
LX

wherein A and B independently are Arg or Leu.

### Sulfonated Amino Acid Complexing Perturbants

Sulfonated modified amino acids, poly amino acids, and peptides are modified by sulfonating at least one free amine group with a sulfonating agent which reacts with at least one of the free amine groups present.

Special mention is made of compounds of the formula



wherein Ar is a substituted or unsubstituted phenyl or naphthyl;

Y is  $-\text{SO}_2-$ ,  $\text{R}^{24}$  has the formula  $-\text{N}(\text{R}^{26})-\text{R}^{25}-\overset{\text{O}}{\underset{\square}{\text{C}}}-$ , wherein:

$\text{R}^{25}$  is  $\text{C}_1$  to  $\text{C}_{24}$  alkyl,  $\text{C}_1$  to  $\text{C}_{24}$  alkenyl, phenyl, naphthyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) phenyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl) phenyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) naphthyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl) naphthyl, phenyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl), phenyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl), naphthyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) and naphthyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl);

$\text{R}^{25}$  is optionally substituted with  $\text{C}_1$  to  $\text{C}_4$  alkyl,  $\text{C}_1$  to  $\text{C}_4$  alkenyl,  $\text{C}_1$  to  $\text{C}_4$  alkoxy,  $-\text{OH}$ ,  $-\text{SH}$  and  $-\text{CO}_2\text{R}^{27}$  or any combination thereof;

$\text{R}^{27}$  is hydrogen,  $\text{C}_1$  to  $\text{C}_4$  alkyl or  $\text{C}_1$  to  $\text{C}_4$  alkenyl;

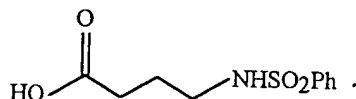
$\text{R}^{25}$  is optionally interrupted by oxygen, nitrogen, sulfur or any combination thereof; and

$\text{R}^{26}$  is hydrogen,  $\text{C}_1$  to  $\text{C}_4$  alkyl or  $\text{C}_1$  to  $\text{C}_4$  alkenyl.

Suitable, but non-limiting, examples of sulfonating agents useful in preparing sulfonated amino acids include sulfonating agents having the formula  $\text{R}^{28}-\text{SO}_2-\text{X}$  wherein  $\text{R}^{28}$  is an appropriate group for the modified amino acid being prepared such as, but not limited to, alkyl, alkenyl, cycloalkyl, or aromatics and X is a leaving group as described above. One example of a sulfonating agent is benzene sulfonyl chloride.

Modified poly amino acids and peptides may include one or more sulfonated amino acid(s). Although linear modified poly amino acids and peptides used generally include only one sulfonated amino acid, other poly amino acid and peptide configurations can include more than one sulfonated amino acid. Poly amino acids and peptides can be polymerized with the sulfonated amino acid(s) or can be sulfonated after polymerization.

Special mention is made of the compound



#### **Modified Amino Acid Derivative Complexing Perturbants**

Modified amino acid, polyamino acid, or peptide derivatives are amino acids, poly amino acids, or peptides which have had at least one acyl-terminus converted to an aldehyde or a ketone, and are acylated at at least one free amine group, with an acylating agent which reacts with at least one of the free amine groups present.

Amino acid, poly amino acid, or peptide derivatives can be readily prepared by reduction of amino acid esters or peptide esters with an appropriate reducing agent. For example, amino acid, poly amino acid, or peptide aldehydes can be prepared as described in an article by R. Chen *et al.*, *Biochemistry*, **1979**, *18*, 921-926. Amino acid, poly amino acid, or peptide ketones can be prepared by the procedure described in Organic Syntheses, Col. Vol. IV, Wiley, (1963), page 5. Acylation is discussed above.

For example, the derivatives may be prepared by reacting a single amino acid, poly amino acid, or peptide derivative or mixtures of two or more amino acid or peptide derivatives, with an acylating agent or an amine modifying agent which reacts with free amino moieties present in the derivatives to form amides. The amino acid, poly amino acid, or peptide can be modified and

subsequently derivatized, derivatized and subsequently modified, or simultaneously modified and derivatized. Protecting groups may be used to avoid unwanted side reactions as would be known to those skilled in the art.

In modified poly amino acid or peptide derivative, one or more of the amino acid may be derivatized (an aldehyde or a ketone) and/or modified, (acylated) but there must be at least one derivative and at least one modification.

Special mention is made of the modified amino acid derivatives N-cyclohexanoyl-Phe aldehyde, N-acetyl-Phe-aldehyde, N-acetyl-Tyr ketone, N-acetyl-Lys ketone and N-acetyl-Leu ketone, and N-cyclohexanoyl phenyl-alanine aldehyde.

### Carboxylic Acid Complexing Perturbants

Various carboxylic acids and salts of these carboxylic acids may be used as complexing perturbants. These carboxylic acids have the formula:



wherein  $R^{29}$  is  $C_1$  to  $C_{24}$  alkyl,  $C_2$  to  $C_{24}$  alkenyl,  $C_3$  to  $C_{10}$  cycloalkyl,  $C_3$  to  $C_{10}$  cycloalkenyl, phenyl, naphthyl, ( $C_1$  to  $C_{10}$  alkyl)phenyl, ( $C_2$  to  $C_{10}$  alkenyl)phenyl, ( $C_1$  to  $C_{10}$  alkyl)naphthyl, ( $C_2$  to  $C_{10}$  alkenyl)naphthyl, phenyl( $C_1$  to  $C_{10}$  alkyl), phenyl( $C_2$  to  $C_{10}$  alkenyl), naphthyl( $C_1$  to  $C_{10}$  alkyl) and naphthyl( $C_2$  to  $C_{10}$  alkenyl);

$R^{29}$  being optionally substituted with  $C_1$  to  $C_{10}$  alkyl,  $C_2$  to  $C_{10}$  alkenyl,  $C_1$  to  $C_4$  alkoxy, -OH, -SH,  $-CO_2R^{30}$ ,  $C_3$  to  $C_{10}$  cycloalkyl,  $C_3$  to  $C_{10}$  cycloalkenyl, heterocyclic having 3-10 ring atoms wherein the hetero atom is one or more atoms of N, O, S or any combination thereof, aryl, ( $C_1$  to  $C_{10}$  alk)aryl, aryl( $C_1$  to  $C_{10}$ )alkyl, or any combination thereof;

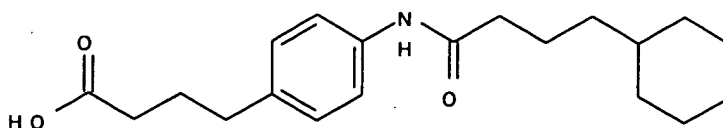
$R^{29}$  being optionally interrupted by oxygen, nitrogen, sulfur, or any combination thereof; and

$R^{30}$  is hydrogen,  $C_1$  to  $C_4$  alkyl or  $C_2$  to  $C_4$  alkenyl.

The preferred carboxylic acids are cyclohexanecarboxylic acid, cyclopentanecarboxylic acid, cycloheptanecarboxylic acid, hexanoic acid, 3-cyclohexanepropanoic acid, methylcyclohexanecarboxylic acid, 1,2-cyclohexanedicarboxylic acid, 1,3-cyclohexanedicarboxylic acid, 1,4-cyclohexanedicarboxylic acid, 1-adamantanecarboxylic acid, phenylpropanoic acid, adipic acid, cyclohexanepentanoic acid, cyclohexanebutanoic acid, pentylcyclohexanoic acid, 2-cyclopentanehexanoic acid, cyclohexane pentanoic acid, hexanedioic acid, cyclohexanebutanoic acid, and (4-methylphenyl) cyclohexane acetic acid.

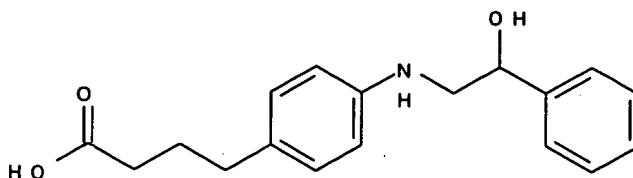
#### Other Examples of Complexing Perturbants

Although all complexing perturbants which can form the supramolecular complexes described herein are within the scope of the present invention, other examples of complexing perturbants include, but are not limited to, 2-carboxymethyl-phenylalanine-leucine; 2-benzyl succinic acid, an actinonin, phenylsulfonyl aminophenyl-butyric acid,



LXIII

and



LXIV

### Mimetics

Mimetics within the scope of the present invention are constructs which are structural and/or functional equivalents of an original entity. Structural and chemically functional mimetics of the supramolecular complexes and the reversible transportable intermediate states of active agents are not necessarily peptidic, as non-peptidic mimetics can be prepared which have the appropriate chemical and/or structural properties. However, preferred mimetics are peptides which have a different primary structure than the supramolecular complex or the intermediate state, but retain the same secondary and tertiary structure of the supramolecular complex or the intermediate state. Although mimetics may have less bioactivity than a native state or intermediate state active agent or supramolecular complex, the mimetics may possess other important properties which may not be possessed by the native state such as, for example, further increased ability to be delivered orally.

Methods of preparation of such mimetics are described, for example, in Yamazaki et al., Chirality 3:268-276 (1991); Wiley et al., Peptidomimetics Derived From Natural Products, Medicinal Research Reviews, Vol. 13, No. 3, 327-384 (1993); Gurrath et al., Eur. J. Biochem 210:991-921 (1992); Yamazaki et al., Int. J. Peptide Protein Res. 37:364-381 (1991); Bach et al., Int. J. Peptide Protein Res. 38:314-323 (1991); Clark et al., J. Med. Chem. 32:2026-2038 (1989); Portoghesi, J. Med. Chem. 34:(6) 1715-1720 (1991); Zhou et al., J. Immunol. 149 (5) 1763-1769 (Sept 1, 1992); Holzman et al., J. Protein Chem. 10: (5) 553-563 (1991); Masler et al., Arch. Insect Biochem. and Physiol. 22:87-111 (1993); Saragovi et al., Biotechnology 10: (July 1992); Olmsteel et al., J. Med. Chem. 36:(1) 179-180 (1993); Malin et al. Peptides 14:47-51 (1993); and Kouns et al., Blood 80:(10) 2539-2537 (1992); Tanaka et al., Biophys. Chem. 50 (1994) 47-61; DeGrado et al., Science 243 (February 3, 1989); Regan et al., Science 241: 976-978 (August 19, 1988); Matouschek et al., Nature 340: 122-126 (July 13, 1989); Parker et al., Peptide Research 4: (6)



347-354 (1991); Parker et al., Peptide Research 4:(6) 355-363 (1991); Federov et al., J. Mol. Biol. 225: 927-931 (1992); Ptitsyn et al., Biopolymers 22: 15-25 (1983); Ptitsyn et al., Protein Engineering 2:(6) 443-447 (1989).

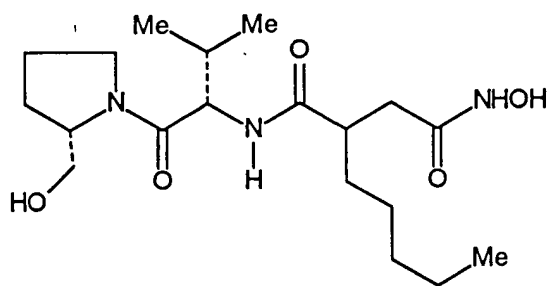
For example, protein structures are determined by the collective intra- and inter-molecular interactions of the constituent amino acids. In alpha helices, the first and fourth amino acid in the helix interact non-covalently with one another. This pattern repeats through the entire helix except for the first four and last four amino acids. In addition, the side chains of amino acids can interact with one another. For example, the phenyl side chain of phenylalanine would probably not be solvent exposed if that phenylalanine were found in a helix. If the interactions of that phenylalanine contributed to helix stability then substituting an alanine for a phenylalanine would disrupt the helix and change the conformation of a protein.

Therefore, a mimetic could be created by first determining which amino acid side chains became solvent exposed and thus removed from contributing to stabilization of the native state such as by the technique of scanning mutagenesis. Mutants containing amino acid substitutions at those same sites could be created so that the substituted amino acids would render the protein conformation more intermediate-like than native-like. Confirmation that the appropriate structure had been synthesized could come from spectroscopy and other analytical methods.

### **Delivery Compositions**

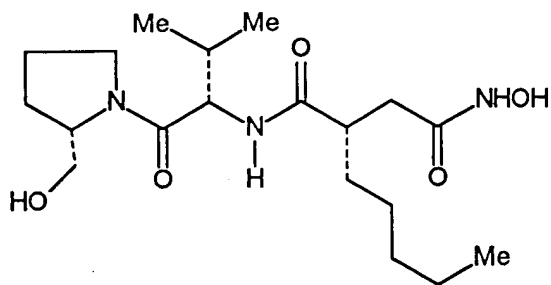
Delivery compositions which include the supramolecular complex described above are typically formulated by mixing an effective amount of perturbant appropriate for the chosen route of delivery i.e, subcutaneous, nasal, or sublingual, with the active agent. The components can be prepared well prior to administration or can be mixed just prior to administration.

The delivery compositions of the present invention may also include one or more enzyme inhibitors. Such enzyme inhibitors include, but are not limited to, compounds such as actinonin or epiactinonin and derivatives thereof. These compounds have the formulas below:



Actinonin

LXV

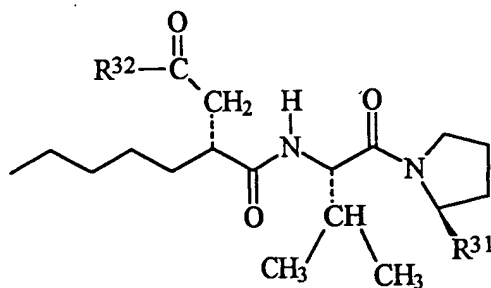


Epiactinonin

LXVI

Derivatives of these compounds are disclosed in U.S. Patent No. 5,206,384.

Actinonin derivatives have the formula:



LXVII

wherein  $R^{31}$  is sulfoxymethyl or carboxyl or a substituted carboxy group selected from carboxamide, hydroxyaminocarbonyl and alkoxycarbonyl groups; and  $R^{32}$  is hydroxyl, alkoxy, hydroxyamino or sulfoxyamino group. Other enzyme inhibitors include, but are not limited to, aprotinin (Trasylol) and Bowman-Birk inhibitor.

The stabilizing additives may be incorporated into a carrier solution of the supramolecular complex. With some drugs, the presence of such additives promotes the stability and dispersibility of the agent in solution.

The stabilizing additives may be employed at a concentration ranging between about 0.1 and 5 % (W/V), preferably about 0.5 % (W/V). Suitable, but non-limiting, examples of stabilizing additives include gum acacia, gelatin, methyl cellulose, polyethylene glycol, carboxylic acids and salts thereof, and polylysine. The preferred stabilizing additives are gum acacia, gelatin and methyl cellulose.

The amount of active agent is an amount effective to accomplish the purpose of the particular active agent. The amount in the composition typically is a pharmacologically or biologically effective amount. However, the amount can be less than a pharmacologically or biologically effective amount when the composition is used in a dosage unit form, such as a capsule, a tablet or a liquid, because the dosage unit form may contain a multiplicity of carrier/biologically or chemically active agent compositions or may contain a divided pharmacologically or biologically effective amount. The total effective amounts can then be administered in cumulative units containing, in total, pharmacologically or biologically or chemically active amounts of biologically or pharmacologically active agent.

The total amount of active agent, and particularly biologically or chemically active agent, to be used can be determined by those skilled in the art. However, it has surprisingly been found that with some biologically or chemically active agents, the use of the presently disclosed carriers provides extremely efficient delivery, particularly in, intranasal, sublingual, or subcutaneous systems.

Therefore, lower amounts of biologically or chemically active agent than those used in prior dosage unit forms or delivery systems can be administered to the subject, while still achieving the same blood levels and therapeutic effects.

The amount of perturbant used is a delivery effective amount.

The compositions of the present invention may be formulated into dosage units by the addition of one or more excipient(s), diluent(s), disintegrant(s), lubricant(s), plasticizer(s), colorant(s), or dosing vehicle(s). Preferred dosage unit forms are oral dosage unit forms. Most preferred dosage unit forms include, but are not limited to, tablets, capsules, or liquids. The dosage unit forms can include biologically, pharmacologically, or therapeutically effective amounts of the active agent or can include less than such an amount if multiple dosage unit forms are to be used to administer a total dosage of the active agent. Dosage unit forms are prepared by methods conventional in the art.

The subject invention is useful for administering biologically active agents to any animals such as birds; mammals, such as primates and particularly humans; and insects. The systems are particularly advantageous for delivering chemical or biologically active agents which would otherwise be destroyed or rendered less effective by conditions encountered before the active agent in the native state reaches its target zone (i.e. the area to which the active agent to be delivered) and by conditions within the body of the animal to which they are administered. Particularly, the present invention is useful in subcutaneously, intranasally, or sublingually administering active agents.

### **DESCRIPTION OF THE PREFERRED EMBODIMENTS**

The following examples illustrate the invention without limitation. All parts and percentages are by weight unless otherwise indicated.

**Example 1 -         $\alpha$ -Interferon Native Gels**

Native gradient gels (Pharmacia) were run with 647  $\mu\text{g}/\text{ml}$  of  $\alpha$ -interferon, (Intron-A - Schering-Plough) and increasing amounts (10-500  $\text{mg}/\text{mL}$ ) of perturbant (mixture of L-Valine, L-Leucine, L-phenylalanine, L-lysine and L-arginine modified with benzenesulfonylchloride) (valine-7.4%, leucine-16.5%, phenylalanine - 40.3%, lysine - 16.2% and arginine - 19.6%). 4 $\mu\text{l}$  of material were loaded onto the gel using a 6/4 comb for loading.

Results are illustrated in Figure 1.

Lane 1 = High molecular weight marker (Bio-Rad) - 1:20 dilution w/dH<sub>2</sub>O - (5 $\mu\text{l}$  - >100 $\mu\text{l}$ ).

Lane 2 =  $\alpha$ -interferon A (647 $\mu\text{g}/\text{mL}$ ) control 5 $\mu\text{l}$  + 5 $\mu\text{l}$  Bromophenol Blue (BPB) - (1.29 $\mu\text{g}$  loaded).

Lane 3 =  $\alpha$ -interferon + perturbant (10 $\text{mg}/\text{mL}$ ) - 50 $\mu\text{l}$   $\alpha$ -interferon + 50 $\mu\text{l}$  BPB = 100 $\mu\text{l}$  (1.29 $\mu\text{g}$  loaded).

Lane 4 =  $\alpha$ -interferon + perturbant (50 $\text{mg}/\text{mL}$ ) 50 $\mu\text{l}$   $\alpha$ -interferon + 50 $\mu\text{l}$  BPB = 100  $\mu\text{l}$  (1.29 $\mu\text{g}$  loaded).

Lane 5 =  $\alpha$ -interferon + perturbant (100 $\text{mg}/\text{mL}$ ) 50 $\mu\text{l}$   $\alpha$ -interferon + 50 $\mu\text{l}$  BPB = 100 $\mu\text{l}$  (1.29 $\mu\text{g}$  loaded).

Lane 6 =  $\alpha$ -interferon + perturbant (500  $\text{mg}/\text{mL}$ ) 5 $\mu\text{l}$   $\alpha$ -interferon + 5 $\mu\text{l}$  BPB = 10 $\mu\text{l}$  (1.29 $\mu\text{g}$  loaded).

**Example 1A -         $\alpha$ -Interferon Native Gradient Gel**

The method of Example 1 was followed substituting the thermal condensation product of glutamic acid, aspartic acid, tyrosine, and phenyl-alanine (Glu-Asp-Tye-Phe) that was fractionated through a 3000 molecular weight cut-off filter for the perturbant.

Results are illustrated in Figure 2.

### Samples

Lane 1 = High Molecular Weight marker (Bio-Rad).

Lane 2 =  $\alpha$ -interferon (647 $\mu$ g/mL) - 5 $\mu$ l + 5 $\mu$ l BPB control.

Lane 3 =  $\alpha$ -interferon + perturbant (10mg/mL) - 50 $\mu$ l + 50 $\mu$ l BPB = 100 $\mu$ l.

Lane 4 =  $\alpha$ -interferon + perturbant - 50 $\mu$ l + 50 $\mu$ l BPB = 100 $\mu$ l.

Lane 5 =  $\alpha$ -interferon + perturbant (100mg/mL) - 50 $\mu$ l Intron A + 50 $\mu$ l BPB = 100 $\mu$ l.

Lane 6 =  $\alpha$ -interferon + perturbant (500mg/mL) - 5 $\mu$ l Intron A + 50 $\mu$ l BPB = 100 $\mu$ l.

Examples 1 and 1A illustrate that  $\alpha$ -interferon alone (lane 2 in Figures 1 and 2) banded at the appropriate molecular weight (approximately 19,000 Daltons). As the amount of perturbant added is increased in each subsequent lane relative to a fixed concentration of  $\alpha$ -interferon, the  $\alpha$ -interferon migrates to a lower, rather than a higher, molecular weight. The change seen with the perturbant of Example 1 is more pronounced than that seen with the perturbant of Example 1A. This indicates that the  $\alpha$ -interferon structure is changing due to the two different perturbants, because if structure were not changing, there would be a shift towards higher molecular weight as perturbant complexes with the active agent.

### Example 2 - Isothermal Titration Calometry

A dosing composition of the perturbant of Example 1 at 2.4mM and sCt at 0.3mM was prepared, and isothermal titration calorimetry was performed at pH 6.5 and 4.5. The buffer at pH 6.5 was 30mM Hepes-30mM NaCl, and the buffer at pH 4.5, was 30mM sodium acetate-30mM NaCl.

All experiments were performed at 30°C using 8.0mM perturbant in the dropping syringe and 1.0mM calcitonin in the calorimeter cell. In all experiments, 15x10 $\mu$ l increments of perturbant were added in 10 second duration additions with 2 minutes equilibration between additions.

Results were validated in experiments where perturbant (8mM) was placed in the dropping syringe, and equivalent increments were added to pH 4.5 buffer (no sCt) and where perturbant was placed in the dropping syringe and 10 $\mu$ l increments were added to pH 6.5 buffer (no sCt). Titration curves were not obtained in these experiments, and the results showed that heat of mixing and/or dilution of perturbant is negligible. Therefore, the experimental isotherms were not corrected by background subtraction.

Results are illustrated in Table 1 below.

### Example 2A

The method of Example 2 was followed substituting the perturbant of Example 1A. Results were validated in experiments where perturbant was placed in the dropping syringe, and equivalent increments were added to pH 4.5 buffer (no sCt).

Results are illustrated in Table 1 below.

TABLE - 1				
Binding Parameters of Perturbants as Determined by ITC <sup>1</sup>				
	K <sub>D</sub> (M)	ΔH (cal/mol)	ΔS (cal/mol °K)	N
<b>pH 6.6</b>				
Example 4	4.59 x 10 <sup>-8</sup>	+ 240	+ 34.4	0.6
Example 4A	6.99 x 10 <sup>-9</sup>	+ 277	+ 38.3	11.6
<b>pH 4.5</b>				
Example 4	Precipitates			
Example 4A	1.29 x 10 <sup>-4</sup>	+ 553	+ 19.8	+ 0.8

### **Example 3 - GuHCl Denaturation Of α-Interferon**

A stock solution of 9.1 mg/mL of α-interferon (Schering Plough Corp.) in 20mM sodium phosphate buffer at pH 7.2 was prepared. Samples were prepared by diluting the α-interferon with the sodium phosphate buffer and 10 M guanidine hydrochloride (GuHCl) (Sigma Chemical Co. - St. Louis, MO) stock solution to 200ug/mL concentration of α-interferon at various concentrations of GuHCl. Diluted samples were allowed to come to equilibrium by incubation for approximately 30 minutes at room temperature prior to measurement.

Fluorescence measurements were made at 25°C using a Hitachi F-4500. Protein tryptophan fluorescence was observed at an excitation wavelength of 298nm and an emission wavelength of 343nm. ANS (1-anilinonaphthalene-8-

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<sup>1</sup> Calorimetry experiments were performed essentially as detailed by You, J.L., Scarsdale, J.N., and Harris, R.B., J. Prot. Chem. 10: 301-311, 1991; You, Junling, Page, Jimmy D., Scarsdale, J. Neel, Colman, Robert W., and Harris, R.B., Peptides 14: 867-876, 1993; Tyler-Cross, R., Sobel, M., Soler, D.F., and Harris, R.B., Arch. Biochem. Biophys. 306: 528-533, 1993; Tyler-Cross, R., Sobel, M., Marques, D., and Harris, R.B., Protein Science 3: 620-627, 1994.



sulfonate) fluorescence was observed at an excitation wavelength of 355nm and an emission wavelength of 530nm. For all fluorescence measurements, a 5nm spectral bandpass was chosen for both excitation and emission.

Results are illustrated in Figure 3.

**Example 4 - Concentration Effect of GuHCl on  $\alpha$ -Interferon Configuration**

GuHCl 5M stock solution was prepared using 20 mM sodium phosphate, pH 7.2 buffer. After dilution, the pH of the stock was checked and adjusted by concentrated HCl. To determine the concentration of final solution the refractive index referenced in Methods in Enzymology, Vol. 6, page 43 by Yasuhiko Nozaki was used.

$\alpha$ -interferon stock (9.1 mg/mL) was mixed with sufficient amounts of GuHCl to yield the concentrations of Table 1A below:

**Table 1A -  $\alpha$ -Interferon/GuHCl Solutions**

GuHCl (M)	$\alpha$ -IFN (mg/mL)
0.5	0.60
1.0	0.53
1.5	0.60
2.0	0.50
3.0	0.60
4.0	0.50

Differential scanning calorimetry (DSC) was run, and results are illustrated in Figure 4.

**Example 5 - pH Titration of Intron A as Measured by Intrinsic Tryptophan Fluorescence**

A stock solution of 9.1 mg/mL  $\alpha$ -interferon in 20mM sodium phosphate buffer at pH 7.2 (Schering Plough Corp.) was prepared. Samples were prepared by diluting the  $\alpha$ -interferon to a concentration of 200 ug/mL into solution buffered at various pH values using the following buffers: Glycine at pH 2 and 12, sodium phosphate at pH 3, 4, 5, 7, and boric acid at pH 8. These buffers were prepared as described in the Practical Handbook of Biochemistry and Molecular Biology, Edited by Gerald D. Fasman, 1990. Diluted samples were allowed to come to equilibrium by incubation for approximately 30 minutes at room temperature prior to measurement.

Fluorescence was measured according to the procedure of Example 3. Results are illustrated in Figure 5.

**Example 6 - pH Titration of Insulin Measured by ANS Fluorescence**

A stock solution was prepared by dissolving 2mg of insulin in 1mL of deionized water. 1-anilinonaphthalene-8-sulphonate (ANS) stock solution was prepared by dissolving 10mg in 10mL of deionized water. Samples were prepared by diluting the insulin to a concentration of 200 ug/mL into solution buffered at various pH values using the following buffers: Glycine at pH 2 and 12, sodium phosphate at pH 3, 4, 5, 7, and boric acid at pH 8. These buffers were prepared as described in the Practical Handbook of Biochemistry and Molecular Biology, Edited by Gerald D. Fasman, 1990. The final ANS concentration was 90ug/mL. Diluted samples were allowed to come to equilibrium by incubation for approx. 30 minutes at room temperature prior to measurement.

Fluorescence was measured according to the procedure of Example 3. Results are illustrated in Figure 6.

**Example 7 - Reversibility of Circular Dichroism Spectra of  $\alpha$ -Interferon at pH 2 and 7.2**

Circular dichroism spectra of  $\alpha$ -interferon were generated at pH 7.2. The pH of the solution was then readjusted to pH 2, and the sample was rescanned. The sample solution was then readjusted to 7.2 and rescanned.

Concentration of  $\alpha$ -interferon was 9.2 $\mu$ M or 0.17848mg/mL, ([IFN] stock = 9.1mg/mL). Buffers used were 20mM NaPhosphate at pH 7.2; and 20mM Glycine at pH 2.0.

Reversal of the pH to 7.2 resulted in complete restoration of the native structure, demonstrating the reversibility of the intermediate state. It is believed that the free energy difference between the native state and the intermediate state is small.

Results are illustrated in Figures 7A and 7B.

**Example 8 - Circular Dichroism Spectra of  $\alpha$ -Interferon at 7.2 - pH Dependence**

The extent of ordered secondary structure of  $\alpha$ -interferon at different pH's was determined by circular dichroism (CD) measurements in the far UV range. The large dilution factor of interferon stock ( $\sim 50$  times) resulted in the sample being at the proper pH. Concentration of  $\alpha$ -interferon was  $9.2\mu\text{M}$  or  $0.17848\text{mg/mL}$ , ([IFN] stock =  $9.1\text{mg/mL}$ ). Buffers used were  $20\text{mM}$  sodium phosphate at pH 6.0 and 7.2;  $20\text{mM}$  NaAc at pH 3.0, 4.0, 4.5, 5.0 and 5.5; and  $20\text{mM}$  Glycine at pH 2.0

The secondary structure content was estimated with several fitting programs, each of which decomposes the CD curve into four major structural components:  $\alpha$ -helix,  $\beta$ -sheet, turns, and random coil. Two of those programs were provided with the CD instrument as an analysis facility. The first program uses seven reference proteins: Myoglobin, Lysozyme, Papain, Cytochrome C, Hemoglobin, Ribonuclease A and Chymotrypsin. The second uses Yang.REF reference file.

A third program, CCAFAST, uses the Convex Constraint Algorithm and is described in "Analysis of Circular Dichroism Spectrum of Proteins Using the Convex Constraint Algorithm: A Practical Guide". (A. Perczel, K. Park and G.D. Fasman (1992) Anal. Biochem. 203: 83-93).

Deconvolution of the far UV scans over a range of pH volumes (2.0-7.2) indicates significant compaction of the secondary structure at pH 3.5. The near UV scan indicates a disruption of tertiary structure packing, and the far UV scan indicates that there is still significant secondary structure at this pH.

Results are illustrated in Figure 8.

**Example 9 - DSC of Insulin and Increasing Concentrations of GuHCl**

DSC was performed with  $6\text{mg/mL}$  insulin ( $0.83\text{mM}$  assuming a molecular weight of 6,000) in  $50\text{mM}$  phosphate buffer, pH 7.5. Each subsequent

thermogram was corrected by background subtraction of a 0.6M guanidine-phosphate buffer solution.

Insulin was freshly prepared as a concentrated stock solution in 50mM phosphate buffer, pH 7.5, and an appropriate aliquot was diluted in buffer, filtered through a 2 micron PTFE filter, and degassed for at least 20 minutes. The reference cell contained degassed buffer.

Scanning calorimetry was performed using 5mg 0.83mM porcine insulin (MW 6,000) per mL in 50mM phosphate buffer, pH 7.5. All thermograms were performed on a Microcal MC-2 scanning calorimeter equipped with the DA2 data acquisition system operated in the upscale mode at 1° C/min ( up to 90° C), and data points were collected at 20 second intervals. All scans were initiated at least 20 degrees below the observed transitions for the active agent. All thermograms were corrected for baseline subtraction and normalized for the concentration of macromolecule. According to the methods of the Johns Hopkins Biocalorimetry Center, See, for example, Ramsay et al. *Biochemistry* (1990) 29:8677-8693; Schon et al. *Biochemistry* (1989) 28:5019-5024 (1990) 29: 781-788. The DSC data analysis software is based on the statistical mechanical deconvolution of a thermally induced macromolecular melting profile.

The effect of GuHCl on structure was assessed in DSC experiments where individual solutions were prepared in phosphate buffer, pH 7.5, containing denaturant diluted from a 5M stock solution to concentrations ranging for 0.5-2M.

Results are illustrated in Table 2 below.

Table - 2	
DSC of Insulin and Increasing Concentrations of Guanidine Hydrochloride	
	<u>T<sub>m</sub> (C<sub>p</sub>, max)</u> (°C)
Insulin 0.0M GuHCl	78.3
Insulin + 0.5M GuHCl	79.3
Insulin + 1.0M GuHCl	77.5
Insulin + 2.0M GuHCl	69.7
Insulin + 3.0M GuHCl	no transition observed

**Example 10 - Effect of Ionic Strength on the DSC Spectrum of Insulin**

A sample containing 6mg/mL insulin (0.83mM in 50mM phosphate buffer, pH 7.5, containing 0.25, 0.5, or 1.0M NaCl) was used. Thermograms were performed according to the procedure in Example 9 and were corrected by subtraction of a 0.5M NaCl-phosphate buffer blank as described above.

The effect of increasing ionic strength on structure was assessed in DSC experiments where individual solutions were prepared so as to contain NaCl at concentrations ranging from 0.25-3M.

Results are illustrated in Table 3 below.

Table - 3		
Effect of Ionic Strength on the DSC Spectrum of Insulin		
		<u>T<sub>m</sub> (C<sub>p</sub>, max)</u> (°C)
Insulin	0.0M NaCl	78.3
Insulin	+ 0.25M NaCl	80.7
Insulin	+ 0.5M NaCl	80.7
Insulin	+ 1.0M NaCl	80.7

**Example 10A - Effect of Ionic Strength on the DSC Spectrum of rhGh**

The method of Example 9 was followed substituting 5mg/mL recombinant human growth hormone (rhGh) (225  $\mu$ M based on M,22,128 of GH) in 50mM phosphate buffer, pH 7.5 containing either 0.5 or 1.0M NaCl, for the insulin. The thermograms were corrected by subtraction of a 0.5M NaCl-phosphate buffer blank.

Results are illustrated in Table 4 below.

Table - 4		
Effect of Ionic Strength on the DSC Spectrum of rhGh		
	<u>T<sub>m</sub> (C<sub>p</sub>, max)</u> (°C)	<u><math>\Delta H^\circ</math></u> (kcal/mol)
rhGh 0.0M NaCl	75.2	191.0
rhGh + 0.5M NaCl	75.8	89.7
rhGh + 10.0M NaCl	76.5	50.5

**Example 11 - Effect of pH on the DSC Spectrum of rhGH**

5mg/mL rhGh were dissolved in buffer (0.17mM in 50mM phosphate buffer, assuming a molecular weight of 20,000). The pH of the solution was adjusted to the desired value, and all curves were corrected by baseline subtraction.

The effect of pH on structure was assessed by DSC according to the procedure of Example 9 where individual solutions were prepared in phosphate buffer ranging in pH value from 2.0 to 6.0.

Results are illustrated in Table 5 below.

Table - 5		
Effect of pH on the DSC Spectrum of rhGh		
	<u>T<sub>m</sub> (C<sub>p</sub>, max)</u> (°C)	<u>ΔH°</u> (kcal/mol)
pH 2.0	no transition observed	no transition observed
pH 3.0	no transition observed	no transition observed
pH 3.5	no transition observed	no transition observed
pH 4.0	≈73.0	broad transition
pH 5.0	75.0	161
pH 6.0	75.2	191
pH 7.5 (10 mg/mL)	a) 73 b) 75	(a) + (b) = 632

**Example 12 - Effect of GuHCl on the DSC Spectrum of rhGh**

An initial scan of rhGh was performed at 10mg/mL in the absence of GuHCl (0.33mM assuming 20,000 molecular weight). Subsequently, the concentration of rhGh was lowered to 5mg/mL (0.17mM) in 50mM phosphate buffer, pH 7.5 containing varying concentrations of GuHCl. Each subsequent thermogram was corrected by background subtraction of a 0.5M guanidine-phosphate buffer solution.



The thermograms were corrected by subtraction of a 0.5M NaCl-phosphate buffer blank. Scans were performed according to the procedure of Example 9.

Results are illustrated in Table 6 below.

Table - 6			
Effect of Guanidine Hydrochloride on DSC Spectrum of rhGh			
	DOMAIN A <u>T<sub>m</sub> (C<sub>p,max</sub>)</u> (°C)	DOMAIN B <u>T<sub>m</sub> (C<sub>p,max</sub>)</u> (°C)	<u>ΔH°</u> (kcal/mol)
rhGh	72.6	74.3	632
rhGh + 0.5M GuHCl	71.5	not defined, but present	48
rhGh + 1.0M GuHCl	70.9	absent	109
rhGh + 1.5M GuHCl	69.7	absent	12
rhGh + 2.0M GuHCl	70.0	absent	58
rhGh + 2.5M GuHCl	70.7	absent	99

### **Example 13 - pH Dependence of α-Interferon Conformation**

α-interferon stock (9.1 mg/mL) was diluted with buffer to a concentration of 0.6 mg/mL. The sample was dialyzed overnight in buffer (volume ratio of α-interferon to buffer was 1:4000). Since there was no extinction coefficient provided, concentration of the sample used was determined by comparison of absorption spectra of the sample before and after dialysis. For each particular pH, the absorbance of the nondialyzed α-interferon of known concentration was measured at 280nm. Then after dialysis, absorbance was read again to account for the protein loss, dilution, etc. Buffer conditions and α-interferon concentrations were:

pH 3.0: Buffer - 20mM NaAc. [IFN] = 0.50 mg/mL;

pH 4.1: Buffer - 20mM NaAc. [IFN] = 0.53 mg/mL;

pH 5.0: Buffer - 20mM NaAc. [IFN] = 0.37 mg/mL;

pH 6.0: Buffer - 20mM Na Phosphate. [IFN] = 0.37 mg/mL;

pH 7.2: Buffer - 20mM Na Phosphate. [IFN] = 0.48 mg/mL.

DSC scans were performed according to the procedure of Example 9. Although clear, transparent solutions of  $\alpha$ -interferon were obtained for every pH at room temperature, there were noticeable signs of precipitation at pH 5.0 and 6.0 after the temperature scans.

Results are illustrated in Table 7 below.

TABLE - 7		
$\alpha$ -Interferon - pH dependence DSC		
pH	T <sub>m</sub> °C	$\Delta H$ cal/mol
7.2	66.84	732717
6.0	65.34	45580
5.0	67.32	69782
4.1	65.64	60470
3.0	--	--

#### **Example 14 - Concentration Effect of GuHCl on $\alpha$ -Interferon Conformation**

GuHCL/ $\alpha$ -interferon samples were prepared according to the method of Example 4. DSC scans were performed according to the procedure of Example 9.

Results are illustrated in Table 8 below.

TABLE - 8		
$\alpha$ -Interferon in GuHCl DSC		
[GuHCl] M	T <sub>m</sub> °C	$\Delta H$ cal/mol
0.0	67.12	72562
0.5	64.43	50827
1.0	63.04	41705
1.5	60.11	29520
2.0	56.32	24980
3.0	45.90	20577
4.0	--	--

Examples 3-14 illustrate that ionic strength, guanidine hydrochloride concentration, and pH result in changes in the T<sub>m</sub> of active agents, indicating a change in conformation. This was confirmed by fluorescence spectroscopy. The reversible intermediate conformational states can be used as templates to prepare mimetics.

**Example 15 - Preparation of  $\alpha$ -Interferon Intermediate State Mimetics**

An intermediate conformational state of  $\alpha$ -interferon is determined. A peptide mimetic having the secondary and tertiary structure of the intermediate state is prepared.

**Example 16 - Preparation of Insulin Intermediate State Mimetics**

The method of Example 15 is followed substituting an insulin for the  $\alpha$ -interferon.

**Example 17 - Preparation of rhGh Intermediate State Mimetics**

The method of Example 15 is followed substituting recombinant human growth hormone for the  $\alpha$ -interferon.

**Example 18 - Titration of  $\alpha$ -Interferon as Measured by Intrinsic Tryptophan Fluorescence**

A stock solution of 9.1mg/mL  $\alpha$ -interferon in 20mM sodium phosphate buffer at pH 7.2 was prepared. A stock solution of perturbant was prepared by dissolving 800mg of perturbant (L-arginine acylated with cyclohexanoyl chloride) in 2mL of 20mM Sodium Phosphate buffer (pH7).

Samples were prepared by diluting the  $\alpha$ -interferon with the sodium phosphate buffer and perturbant stock solution at various perturbant concentrations. Diluted samples were allowed to come to equilibrium by incubation for approximately 30 minutes at room temperature prior to measurement.

Fluorescence from the endogenous tryptophan resident of  $\alpha$ -interferon were measured according to the procedure of Example 3. The perturbant did not contain a fluoophore.

Results are illustrated in Figure 9.

**Example 19 - Differential Scanning Colorimetry of  $\alpha$ -Interferon and Perturbant**

Perturbant binding DSC was conducted using 20mM NaPhosphate buffer at pH 7.2. Dry perturbant was weighed out to make perturbant stock solutions.  $\alpha$ -interferon stock was diluted in the buffer.  $\alpha$ -interferon solution was not dialyzed prior to experiments for the purpose of having the same active concentration for the whole set.

DSC thermograms were generated with  $\alpha$ -interferon at a concentration of 0.64 mg/ml and a perturbant (phenylsulfonyl-para-aminobenzoic acid purified to >98% (as determined by reverse phase chromatography prior to generation of the spectra)) at perturbant concentrations of 5, 10, 25 and 100 mg/ml. DSC was

conducted on a DASM-4 differential scanning calorimeter interfaced to an IBM PC for automatic collection of the data. The scanning rate was 60 °C/h.

Results are illustrated in Table 9 below and Figure 10.

**Comparative Example 19\* - Different Scanning Calorimetry of  $\alpha$ -Interferon**

The method of Example 19 was followed substituting  $\alpha$ -interferon without perturbant. Results are illustrated in Table 9 below and Figure 10.

TABLE - 9		
$\alpha$ -Interferon + Perturbant DSC		
Perturbant - mg/ml	T <sub>m</sub> °C	$\Delta H$ cal/mol
0	67.12	72562
5	64.37	60151
10	62.3	53161
25	58.15	35393
100	46.18	5439.3

DSC scans where the added concentration of perturbant ranged from 0-100 mg/mL show induced conformational changes in the  $\alpha$ -interferon that occur in a concentration dependent manner. At 100 mg/mL of the perturbant, the thermogram indicated that the  $\alpha$ -interferon Cp vs. T<sub>m</sub> curve was a flat line. The flat Cp vs. T<sub>m</sub> curve obtained at 100 mg/mL of perturbant indicates that hydrophobic residues within the  $\alpha$ -interferon molecule became solvent exposed. It is clear that the perturbant was able to change the structure of  $\alpha$ -interferon in a concentration dependent manner.

**Example 20 - Dialysis Experiments - Reversibility of Complexing with the Perturbant**

An  $\alpha$ -interferon stock solution at a concentration of 9.1 mg/mL was diluted with buffer to an  $\alpha$ -interferon concentration of 0.6 mg/mL. DSC was performed according to the procedure of Example 19.

Results are illustrated in Figure 11A.

$\alpha$ -interferon (0.6 mg/ml) and the perturbant of Example 19 (100 mg/ml) were mixed with no apparent changes in the  $C_p$  of the solution. This solution was then dialyzed overnight into phosphate buffer, and the thermogram was rerun. Results are illustrated in Figure 11B.

The dialyzed sample had essentially the same  $T_m$  and the same area under the  $C_p$  vs.  $T_m$  curve as it did prior to addition of the perturbant. This indicated that not only was the perturbant able to induce conformational changes in the protein but that this process was reversible. Dilution was enough of a driving force to effect disengagement of the perturbant from the active agent.

**Example 21 - Perturbant and  $\alpha$ -Interferon DSC**

The method of Example 4 was followed, substituting the perturbant of Example 19 for the GuHCl.

Results are illustrated in Figure 12.

The DSC experiments on the equilibrium denaturation of  $\alpha$ -interferon indicate the existence of intermediate conformations of the molecule.  $\Delta H$  vs.  $T_m$  plots indicate the energetics of intermediate conformations occupied by  $\alpha$ -interferon at each set of experimental conditions.

**Example 22 - Perturbant Concentration Dependent Change in  $\alpha$ -Interferon**

The method of Example 19 was followed substituting cyclohexanoyl phenylglycine for the perturbant.

Results are illustrated in Table 10 below and in Figure 13.

TABLE - 10		
$\alpha$ -Interferon + Perturbant DSC		
Perturbant mg/ml	T <sub>m</sub> °C	$\Delta H$ cal/mol
0	67.12	72562
5	63.00	42299
10	59.49	43058
25	52.79	27237
100	--	0

Cyclohexanoyl phenylglycine induced conformational changes in  $\alpha$ -interferon that were concentration dependent.

**Example 23 - Perturbant and  $\alpha$ -Interferon DSC**

The method of Example 4 was followed substituting the perturbant of Example 30 for the GuHCl.

Results are illustrated in Figure 14.

The  $\Delta H$  v. T<sub>m</sub> plot indicates the existence of an equilibrium intermediate conformation of  $\alpha$ -interferon that is stable at below 5 and 25 mg/ml of added cyclohexanoyl phenylglycine perturbant.

**Example 24 - Isothermal Titration Calorimetry of  $\alpha$ -Interferon with Perturbant**

Isothermal titration calorimetry of perturbant complexing with  $\alpha$ -interferon was performed at 25°C at two different pH's. The buffers used were 20mM NaPhosphate for pH 7.2 and 20mM NaAc for pH 3.0.  $\alpha$ -interferon solution was dialyzed before the experiment to reach the appropriate pH. Dry perturbants were weighed and diluted in dialysate.

ITC was conducted on a MicroCal OMEGA titration calorimeter (MicroCal Inc. - Northampton, MA). Data points were collected every 2 seconds, without subsequent filtering.  $\alpha$ -interferon solution placed in 1.3625mL cell was titrated

using a 250 $\mu$ L syringe filled with concentrated perturbant solution. A certain amount of titrant was injected every 3-5 minutes for up to 55 injections.

A reference experiment to correct for the heat of mixing of two solutions, was performed identically except that the reaction cell was filled with buffer without active agent.

Analysis of the data was performed using the software developed at the Johns Hopkins University Biocalorimetry Center.

The titration at pH 7.2 included 53 injections of 2 $\mu$ L of the perturbant of Example 30 (50mg/mL = 191.6mM (MW 261)) and  $\alpha$ -interferon (1.3 mg/mL = 0.067mM (MW 19400)).

Results are illustrated in Figure 15.

Curve fitting indicated multiple independent sites:

$n(1) = 121.0354$  where  $n = \#$  of completed perturbant molecules

$\Delta H(1) = 58.5932$  cal/Mole perturbant

$\log_{10} K_a(1) = 2.524834$  where  $K_a =$  association constant

x-axis units are concentration of carrier in mM.

y-axis units represent heat/injection expressed in calories.

At pH 3, complexing resulted in a negative enthalpy.

#### **Comparative Example 24\*** - Isothermal Titration Calorimetry of Perturbant

The method of Example 24 was followed, substituting 53 injections of 2  $\mu$ l for the perturbant (50mg/mL = 191.6mM) [IFN] = 0 mg of Example 22 without active agent.

Example 24 and Comparative Example 24\* illustrate that  $\alpha$ -interferon has a positive enthalpy and a binding constant ( $K_d \approx 10^{-3}$ M).



**Example 25 - Isothermal Titration Calorimetry of  $\alpha$ -Interferon and Perturbant Complexing**

The method of Example 24 was followed substituting the perturbant of Example 19 for the perturbant of Example 22.

The titration at pH 7.2 included two runs of 55 injections each of  $5\mu\text{L}$  of perturbant ( $50\text{mg/mL} = 181\text{mM}$  (FW 277)) and  $\alpha$ -interferon ( $2.31\text{mg/mL} = 0.119\text{mM}$ , (MW 19400)).

Results are illustrated in Figure 16.

Curve fitting indicated multiple independent sites:

$n(1) = 55.11848$  where  $n = \#$  of complex perturbant molecules

$\Delta H(1) = -114.587$  cal/Mole perturbant

$\log_{10} K_a(1) = 2.819748$  where  $K_a =$  association constant

x-axis units are concentration of carrier in mM.

y-axis units represent heat/injection expressed in calories.

Complexing of perturbant to  $\alpha$ -interferon at pH 3.0 resulted in precipitation of the complex out of the solution. Due to the heat effect produced by this process, it was impossible to measure the complexing parameters.

**Comparative Example 25\* - Isothermal Titration Calorimetry of Perturbant**

The method of Example 25 was followed, substituting 55 injections of  $5\mu\text{L}$  of the perturbant of Example 26 ( $50\text{mg/mL} = 181\text{mM}$ ) in 20 mM sodium phosphate pH 7.2 without active agent.

The perturbant of Example 19 complexed with  $\alpha$ -interferon resulted in a negative enthalpy and a comparable binding constant to that of the perturbant of Example 22 and  $\alpha$ -interferon.

Examples 24 and 25 indicate that the stronger the perturbant complexes with the active agent and the more thermodynamically stable the intermediate state of the active agent, the greater the bioavailability of the active agent.

Therefore, by plotting the  $\Delta H$  v.  $T_m$  curve for an active agent and a perturbant, those perturbants that induce little or no enthalpic change over the broadest range of  $T_m$  would be preferred perturbants. It is believed that perturbants that stabilize the intermediate state to a greater extent will result in more efficient delivery of the active agent.

**Example 26 - Comparison of the Effects of Three Perturbants On  $\Delta H$  vs.  $T_m$  Plots with  $\alpha$ -Interferon**

DSC experiments were carried out according to the procedure of Example 19, with 0.5 mg/ml  $\alpha$ -interferon mixed with (1) benzoyl para-amino phenylbutyric acid, (2) the perturbant of Example 22, or (3) the perturbant of Example 19.

Benzoyl para-amino phenylbutyric acid was poorly soluble under the buffer conditions. Maximum concentration at which the solution was still transparent at room temperature was  $\sim 8$ mg/mL. Therefore, the concentrations of the perturbant used were 2, 4, and 6mg/mL. Results are illustrate in Figures 17 and 18. The dashed line in Figure 17 represents the linear least squares, and the regression equation is at the top of Figure 17.

$$Y = -1.424e^5 + 3148.8x$$

$$R = 0.9912$$

Figures 17 and 18 illustrate that conformational changes in  $\alpha$ -interferon are more readily produced by benzoyl para-amino phenylbutyric acid than by the perturbants of Examples 22 and 19, and that such changes are more readily produced by the perturbant of Example 22 than by the perturbant of Example 19.

**Example 27 - Isothermal Titration Calorimetry of  $\alpha$ -Interferon and Complexing**

ITC was performed according to the method of Example 24 with 40 injections of 5  $\mu$ L of the perturbant benzoyl para-amino phenylbutyric acid (7.5mg/mL = 24.59mM, (FW 305)) and  $\alpha$ -interferon (2.5mg/mL = 0.129mM, (MW 19400)).

Results are illustrated in Figure 19.

Curve fitting indicated multiple independent sites:

$n(1) = 23.69578$  where  $n = \#$  of complexed perturbant molecules

$\Delta H(1) = 791.5726$  cal/Mole perturbant

$\log 10 K_a(1) = 3.343261$  where  $K_a =$  association constant

x-axis represents concentration of carrier in mM.

y-axis represents heat/injection expressed in calories.

### **Comparative Example 27\***

ITC was performed according to the method of Example 27 with 40 injections of 5  $\mu$ L of the perturbant benzoyl para-amino phenylbutyric acid (7.5mg/mL = 24.59mM) in 20mM NaPhosphate pH 7.2 buffer, without active agent.

The apparent dissociation constant for the perturbant of Example 26 is greater than that for the perturbant of Example 30 ( $10^{-4}$ M) at pH 7.

Therefore, benzoyl para-amino phenylbutyric acid complexes more strongly to  $\alpha$ -interferon and induces the native state-reversible intermediate conformational state at lower concentrations of perturbant.

### **Examples 28-30 - Isothermal Titration Calorimetry of rhGH at pH 7.5 and 4.0 with Different Perturbants**

The ability of rhGh to complex with various perturbants was assessed by ITC using a Microcal Omega titrator, usually equilibrated at 30°C. The sample cell of the calorimeter was filled with degassed rhGH (usually at 0.25mM) prepared in 50mM phosphate buffer, pH 7.5 or 4.0. The perturbant (cyclohexanoyl chloride modified L-tyrosin (a), salicyloyl modified L-phenylalanine (b), or phenylsulfonyl-para-amino benzoic acid (c)) was then placed in the dropping syringe at 1mM (for pH 7.5)

and 2.5mM, (for pH 4.0). Twenty to twenty-five  $10\mu\text{l}$  injections were made into rapidly mixing (400 rpm) solution with 2 minute intervals between injections.

Initial concentration of perturbant placed in the calorimeter sample cell assumed a formula weight of 200 for each perturbant. The pH of each solution was checked after dissolution, but no adjustments of the pH were required. All experiments were performed at  $30^{\circ}\text{C}$ . Initial concentration of rhGh placed in the dropping syringe assumed a molecular weight of 20,000 for rhGh. The pH of each solution was checked after dissolution, but no adjustment of the pH was required.

The heats of reaction were determined by integration of the observed peaks. To correct for heat of mixing and dilution, a control experiment was also performed under identical conditions where aliquots of the test perturbant or rhGh were added to buffer solution only. The sum total of the heat evolved was plotted against the total perturbant concentration to produce the isotherm from which the association constant ( $K_A$ , M), enthalpy change ( $\Delta H$ , kcal/mol), entropy change ( $\Delta S$  (eu), and N, and the stoichiometry of perturbant molecules complexed per equivalent of complexed supramolecular complex, were determined by curve-fitting the binding isotherm against the binding equation described for perturbant complexing in a supramolecular complex possessing one set of independent perturbant complexing sites. The data were deconvoluted using the nonlinear least squares algorithm supplied in the software of the manufacturer.

Results are illustrated in Table 11 below.

Table - 11					
Isothermal Titration Calorimetry of rhGH at pH 7.5 and 4.0 with Different Perturbants					
Perturbant	rhGh (mM)	K <sub>D</sub> (M)	ΔH (kcal/mo l)	ΔS (eu)	N
<b>pH 7.5</b>					
A at 0.25mM	1.0	9.88x10 <sup>-5</sup>	+ 1.4	+ 23.5	7.0
B at 0.25mM	1.0	1.11x10 <sup>-6</sup>	+ 2.1	+ 35.0	0.7
C at 0.25mM	1.0	1.11x10 <sup>-9</sup>	+ 0.8	+ 44.0	10.0
<b>pH 4.0</b>					
A at 0.25mM	1.0	7.81x10 <sup>-5</sup>	-1.5	- 5.6	2.3
B at 0.25mM	1.0	1.61x10 <sup>-9</sup>	-35.6	-90.0	155.9
C at 0.25mM	1.0	2.67x10 <sup>-8</sup>	-1.2	-30.0	122.0

A = cyclohexanoyl chloride modified L-tyrosine

B = salicyloyl modified L-phenylalanine

C = phenylsulfonyl-para-aminobenzoic acid

The positive ΔS values at pH 7.5 indicate that complexing at this pH results in structural change.

#### **Examples 31 and 32 - Pancreatin Inhibition Assay with α-Interferon and Perturbants**

The assay for pancreatin activity was prepared as follows: 0.1mL of a stock solution of α-interferon (9.1mg/mL, 20mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2) (Schering-Plough Corp.) was added to 2.5mL of either phenylsulfonyl-para-aminobenzoic acid perturbant (46) or cyclohexanoyl phenylglycine perturbant (47) (200mg/mL) in 5mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.0. Incubation was carried out at 37°C for 30 and 60 minutes following the addition of 0.1mL of USP pancreatin (20mg/mL) (Sigma Chemical Co.)

0.1mL aliquots were withdrawn at those times points. Enzyme reactions were stopped by the addition of protease inhibitors (Aprotinin and Bowman-Birk Inhibitor (BBI), each at 2mg/mL) and were diluted five-fold to quantitate  $\alpha$ -interferon left intact. A reverse phase HPLC method using a Butyl C-4 cartridge (3.0x0.46cm, Rainin) and employing gradient elution between 0.1% TFA/water and 90% ACN in 0.1% TFA coupled with UV detection at 220nm was used for separating and quantitating  $\alpha$ -interferon. The  $\alpha$ -interferon at 0 minutes was quantitated from an aliquot prior to the addition of pancreatin and was taken to be 100%.

Results are illustrated in Figure 20.

Examples 31 and 32 illustrate that both supramolecular complexes resisted enzymatic degradation. However, in additional testing no correlation was shown between the enzyme inhibitors potency and the ability to deliver drug.

#### **Example 33 - DSC of Heparin at pH 5.0**

DSC thermograms of heparin at pH 5.0 were conducted according to the method of Example 9 using pH, GuHCl, and ionic strength as perturbants.

Thermograms were corrected by subtraction of a heparin .05M NaCl - phosphate buffer blank, but an individual blank was not used for each NaCl concentration.

Results are illustrated in Tables 12-14 below and in Figure 21.

TABLE - 12			
Effects of pH on the DSC Spectrum of 20 $\mu$ g/ml Heparin in 50 mM Phosphate Buffer			
	T <sub>m</sub> (C <sub>p,max</sub> )	$\frac{\Delta H}{\text{(kcal/mol)}}$	$\Delta H_{\text{vH}}$ (kcal/mol)
pH 6.0	62.5	232.1	13.8
pH 6.5 (a)	62.7	213.9	56.8
(b)	71.8	751.9	
pH 7.0 (a)	47.1	187.1	27.6
(b)	72.9	136.4	
pH 7.5	66.2	499.4	83.8

(a) = a domain

(b) = b domain

TABLE - 13			
Effects of 10M Guanidine Hydrochloride in 50mM Phosphate Buffer on the DSC Spectrum of Heparin			
	T <sub>m</sub> (C <sub>p,max</sub> )	$\frac{\Delta H}{\text{(kcal/mol)}}$	$\Delta H_{\text{vH}}$ (kcal/mol)
heparin	67.2	499.4	83.8
heparin + 0.5M GuHCl	50.5	287.3	170.9
heparin + 1.0M GuHCl	60.5	415.0	97.1
heparin + 1.5M GuHCl	---	1716.5	24.3
heparin + 2.0M GuHCl	---	2533.7	19.2

TABLE - 14				
Effect of Ionic Strength on the DSC Spectrum of 20 $\mu$ g/ml of Heparin in 50mM Phosphate Buffer pH 7.0				
	T <sub>m</sub> (C <sub>p,max</sub> )		$\frac{\Delta H}{\text{(kcal/mol)}}$	$\Delta H_{vH}$ (kcal/mol)
0.0 M NaCl	47.1	187.1	72.9	136.4
0.25M NaCl	46.1	0.112	not present	
0.50M NaCl	41.6	0.094	not present	
0.75M NaCl	27.5	0.00	not present	
1.0 M NaCl	no transition observed			

These data indicate that non-proteinaceous active agents are able to change conformation in response to a perturbant.

#### Example 34 - Column Chromatography of Heparin and Perturbants

The following materials were used:

##### Column:

10mm x 30cm, low pressure, glass column from Pharmacia w/adjustable bed volume. The bed volume used was 22 cm at a pressure of 0.8 Mpa.

##### Packing:

Heparin covalently bonded to Sepharose CL-6B with no linker molecule.

Sepharose fractionation range: 10, 000 - 4,000,000.

The density of heparin was 2mg/cc as per Pharmacia Q.C. Department.

##### Conditions:

The mobil phase was 67mM phosphate buffer, pH7.4.

The flow rate was 1.5mL/min isocratic.

The run time was 45 minutes.



Sample detection was done with a Perkin Elmer refractive index detector.

Column integrity was confirmed by injecting protamine and observing a retention time greater than 1 hour. Void volume was determined by injecting water and measuring time of elution.

Each of the perturbants of Table 15 below (5mg) was independently dissolved in 1mL of mobil phase and injected (100ul) into the column. Time of elution was measured.  $K'$  value was determined by using the following equation (as per USP):

$$K' = (\text{Ret. time Carrier} / \text{Ret. time Water}) - 1$$

The results were compared between each perturbant as well as their respective *in vivo* performance in Figure 29.  $K'$  (the degree of retardation) values in the figure have been corrected by subtraction of the  $K'$  value determined from the sepharose column from the  $K'$  value determined from the heparin-sepharose column.

Table - 15	
PERTURBANTS	
cyclohexylidenebutyric acid (2)-Na salt	#1
cylcohexanebutyroyl (2-) aminobutyric acid (4)	#2
phenylacetyl-para-aminobutyric acid	#3
ortho-methylcyclohexanoyl - aminobutyric acid (4)	#4
phenylacetyl-aminohexanoic acid (6-)	#5
cinnamoyl-para-aminophenylbutyric acid	#6
cyclohexanebutyroyl (2-) - para-aminophenylbutyric acid	#7
hydrocinnamoyl - para-aminophenylbutyric acid	#8
cyclohexanebutyroyl (2-)-leu-leu	#9
cyclohexanebutyroyl (2-)-gly	#10

**Example 35 - Comparison of the Effects of Six Perturbants On  $\Delta H$  vs.  $T_m$  Plots with DPPC**

DSC experiments were carried out according to the procedure of Example 19, with 1.0 mg/ml dipalmitoylphosphatidylcholine (DPPC) mixed with perturbants XI, L, LII, LIII, and LIV. The concentrations of the perturbant were varied from 0 to 20 mg/ml.

Results are illustrated in Figure 22.

**Example 36 - Differential Scanning Colorimetry of DPPC and Perturbant Compound L**

Perturbant binding DSC was conducted using 20mM NaPhosphate buffer at pH 7.2. Dry perturbant L was weighed out to make perturbant stock solutions. DPPC stock solution was prepared in the buffer.

DSC thermograms were generated with DPPC at a concentration of 1.0 mg/ml. The perturbant concentrations used were 0, 5, 10, and 20 mg/ml. The DSC was performed as described in Example 19.

Results are illustrated in Figure 23.

**Example 37 - Differential Scanning Colorimetry of DPPC Perturbant Compound L and rhGH**

Perturbant binding DSC was conducted using 20mM NaPhosphate buffer at pH 7.2. Dry perturbant L was weighed out to make perturbant stock solutions. DPPC stock solution was prepared in the buffer. rhGH solution was prepared as described in Example 10A.

DSC thermograms were generated with DPPC at a concentration of 1.0 mg/ml. Samples having DPPC alone; DPPC with 10 mg/ml of perturbant; DPPC with 0.3 mg/ml of rhGH; and DPPC, 10 mg/ml of perturbant and 0.3 mg/ml of rhGH were prepared and analyzed. The DSC was performed as described in Example 19.

Results are illustrated in Figure 24.

**Example 38 - Differential Scanning Colorimetry of DPPC Perturbant Compound LII and rhGH**

Perturbant binding DSC was conducted using 20mM NaPhosphate buffer at pH 7.2. Dry perturbant LII was weighed out to make perturbant stock solutions. DPPC stock solution was prepared in the buffer. rhGH solution was prepared as described in Example 10A.

DSC thermograms were generated with DPPC at a concentration of 1.0 mg/ml. Samples having DPPC alone; DPPC with 5 mg/ml of perturbant; DPPC with 0.3 mg/ml of rhGH; and DPPC, 5 mg/ml of perturbant and 0.3 mg/ml of rhGH were prepared and analyzed. The DSC was performed as described in Example 19.

Results are illustrated in Figure 25.

**Example 39 - Differential Scanning Colorimetry of DPPC Perturbant Compound XI and rhGH**

Perturbant binding DSC was conducted using 20mM NaPhosphate buffer at pH 7.2. Dry perturbant XI was weighed out to make perturbant stock solutions. DPPC stock solution was prepared in the buffer. rhGH solution was prepared as described in Example 10A.

DSC thermograms were generated with DPPC at a concentration of 1.0 mg/ml. Samples having DPPC alone; DPPC with 2 mg/ml of perturbant; DPPC with 0.3 mg/ml of rhGH; and DPPC, 2 mg/ml of perturbant and 0.3 mg/ml of rhGH were prepared and analyzed. The DSC was performed as described in Example 19.

Results are illustrated in Figure 26.

**Example 40 - Dynamic Light Scattering of Compound L**

Solutions of compound L were prepared in a 10 mM phosphate buffer at a pH of 7.0. The concentrations tested were 10, 15, and 20 mg/ml. The solutions were analyzed using standard microscopic light scattering techniques.

Results are illustrated in Figure 27.

**Examples 41 and 42 - *In Vivo* Evaluation of Recombinant Growth Hormone in Rats**

Dosing compositions were prepared by mixing the modified amino acids and recombinant human growth hormone (rhGH) as listed in Table 16 below in a phosphate buffer solution at a pH of about 7-8.

Rats were administered the dosing composition by sublingual or intranasal administration. Delivery was evaluated by using an ELISA assay for rhGH from Medix Biotech, Inc. For intracolonic administration, a sample was prepared and dosed to fasted rats at 25 mg/kg of carrier in a buffered solution containing propylene glycol (0-50%) and 1 mg/kg rhGH.

Results are illustrated in Table 16 below.

**Comparative Example 41A**

rhGH (6 mg/ml) was administered by oral gavage to a rat, and delivery was evaluated according to the procedure of Example 41A.

Results are illustrated in Table 16 below.

Table 16					
<i>In Vivo</i> Delivery of rhGH					
Example	Carrier	Carrier Dose (mg/kg)	Drug Dose (mg/kg)	Method of Administration	Mean Peak Serum Levels of rhGH (ng/mL)
42A	none	0	6	oral	< 10 +/- 10
41	XIX-1	100	3	sublingual	119.14 +/- 65.6
42	XIX-1	25	1	intranasal	92.7 +/- 73.2

### **Examples 43-54 - *In Vivo* Evaluation of Recombinant Growth Hormone in Rats**

#### **Preparation of Dosing solutions.**

The delivery agents were reconstituted with distilled water and adjusted to pH 7.2-8.0 with either aqueous hydrochloric acid or aqueous sodium hydroxide. A stock solution of rhGH was prepared by mixing rhGH, D-mannitol and glycine and dissolving this mixture in 2% glycerol/water. The stock solution was then added to the delivery agent solution. Several delivery agent to active agent ratios were studied.

#### ***In vivo* experiments.**

Male Sprague-Dawley rats weighing 200-250g were fasted for 24 hours and administered ketamine (44 mg/kg) and chlorpromazine (1.5 mg/kg) 15 minutes prior to dosing. The rats were administered one of the dosing solutions described above by subcutaneous injection, intranasal instillation, or sublingual instillation. Blood samples were collected serially from the tail artery for serum calcium concentration determination or serum rhGH concentrations. The dose of rhGH administered in these experiments was 0.1 mg/kg.

Serum rhGH concentrations were quantified by an rhGH enzyme immunoassay test kit. The results are given in Table 17 and Figures 28 and 29.

In Figure 29 the circles represent the response following SL dosing of an aqueous solution of compound CXXIII-H and rhGH. The squares represent the

response following IN dosing of an aqueous solution of compound CXXIII-H and rhGH. The triangles represent the response following IC dosing of an aqueous solution of compound CXXIII-H and rhGH. The dose of compound CXXIII-H was 25 mg/kg and the dose of rhGH was 1 mg/kg.

#### **Comparative Example 43A**

rhGH (1 mg/kg) was administered by oral gavage to a rat, and delivery was evaluated according to the procedure of Example 43.

Results are illustrated in Table 17 below.

Table 17

Table 17			
Delivery Agent Enhancement of Recombinant Human Growth Hormone (rhGH) Bioavailability Administered by Subcutaneous Administration.			
Example	Deliver Agent	Delivery Agent Dose (mg/kg)	Peak Serum [rhGH] (ng/mL)
43	CXXIII-H	1.0	22 ± 3
43A	None	0.0	4 ± 2
44	CXXIII-H	2.5	25 ± 5
45	CXXIII-H	25	30 ± 6
46	CXI-G	2.5	16 ± 2
47	LVIII-A	1.0	29 ± 10
48	LXXXVI-B	1.0	22 ± 7
49	LXXXVI-B	2.5	23 ± 5
50	LXI-A	2.5	26 ± 5
51	CX-1	1.0	15 ± 3
52	CXV-G	1.0	25 ± 3
53	LXVI-A	1.0	33 ± 5
54	CIX-1	1.0	16 ± 3

#### **Examples 55-60 - *In Vivo* Evaluation of Salmon Calcitonin in Rats**

##### **Preparation of Dosing solution.**

The delivery agents were reconstituted with distilled water and adjusted to pH 7.2-8.0 with either aqueous hydrochloric acid or aqueous sodium hydroxide. A stock solution of sCT was prepared by dissolving sCT in citric acid (0.085N). The stock solution was then added to the delivery agent solution. Several different delivery agent to active agent ratios were studied.

*In vivo experiments.*

Male Sprague-Dawley rats weighing 200-250g were fasted for 24 hours and administered ketamine (44 mg/kg) and chlorpromazine (1.5 mg/kg) 15 minutes prior to dosing. The rats were administered one of the dosing solutions described above by subcutaneous injection. Blood samples were collected serially from the tail artery for serum calcium concentration.

Serum calcium concentrations were quantified by the o-cresolphthalein complexone method (Sigma) using a UV/VIS spectrophotometer (Perkin Elmer). The results are given in Table 5.

**Examples 55A**

Salmon calcitonin was administered by oral gavage to rats, and delivery was evaluated according to the Procedure of Example 55. The results are given in Table 18 below.

Table 18			
Delivery Agent Enhancement of Salmon Calcitonin (sCT, dosed at 0.2 $\mu$ g/kg) Bioavailability Administered by Subcutaneous Administration.			
Example	Deliver Agency	Delivery Agent Dose ( $\mu$ g/kg)	Percent Decrease in Serum Calcium
55	CXXIII-H	2	17 $\pm$ 3
55A	None	0	17 $\pm$ 2
56	CXXIII-H	20	25 $\pm$ 4
57	CXXIII-H	200	25 $\pm$ 5
58	CXXIII-H	2000	26 $\pm$ 5
59	CXI-G	20	21 $\pm$ 4
60	CXIV-G	20	20 $\pm$ 3



All patents, applications, test methods, and publications mentioned herein are hereby incorporated by reference.

Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed disclosure. All such modifications are within the full extended scope of the appended claims.

100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000